An Investigation into the Anti-tumour Properties and Underlying Mechanisms of Natural Polyphenols Against Ovarian Cancer.

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iv. Abstract

Ovarian cancer is the deadliest gynaecologic cancer in New Zealand. Its high mortality rate is due to the fact that it is usually diagnosed at an advanced stage. Advanced ovarian cancer is less responsive to current cytotoxic treatment. Thus, there is an urgent need for novel anti-cancer drugs that can improve patient longevity and quality of life. One of the clinical features of advanced ovarian cancer is the growth of secondary tumours due to the highly metastatic nature of the disease. Cancer cells disseminate from the ovary, some form cell clusters that travel through the abdominal cavity by physiological movement of body fluid and then deposit on the abdominal wall and internal organs to generate secondary tumours. The exact mechanisms of how these cells metastasize are unclear, but prognosis typically worsens if levels of vascular endothelial growth factor (VEGF) are elevated. This study investigated the anti-tumour activities of naturally occurring food compounds resveratrol, acetyl resveratrol and (-)-Epicatechin-3-gallate (EGCG), in cell spheroids/clusters of ovarian cancer. It also examined the protein expression of various proteins involved in the NF-κB signalling pathway. This pathway has been suggested to mediate the secretion of VEGF and is a possible target for the naturally occurring compounds. Results show that resveratrol and acetyl resveratrol reduce cell growth and cellular metabolism in a dose-, time- and cell line- dependent fashion. In addition, the reduction of VEGF is also dose-, time- and cell line- dependent. Paradoxically, another angiogenic protein interleukin-8 (IL-8) secretion is increased. Resveratrol and acetyl resveratrol attenuate the expression of NF-κB but this effect is cell line specific. EGCG has limited effect on cell growth, cellular metabolism and the secretion of VEGF and IL-8. These findings suggest that resveratrol and its derivative may have the ability to supress the angiogenic activity of ovarian cancer cells and warrant further *in vivo* study.

v. Abbreviations.

2D	Two-dimensional
3D	Three-dimensional
Acetyl resveratrol	3,5,4’-tri-O-acetylresveratrol
AP-1	Activator protein 1
Bay 117085	NF-κB inhibiting compound
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
Cisplatin	*cis*-diamminedichloroplatinum(II)
Crystal violet	(tris(4-(dimethylamino)phenyl)methylium chloride
DMEM/F-12	Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immnosorbent assay
ER	Estrogen receptor
EGCG	(-)-Epicatechin-3-gallate
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IKK	IκB kinase
IL-8	Interleukin-8
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium working media
NF-κB	Nuclear factor kappa B
OVCAR-5	Human ovarian adenocarcinoma cell line
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
pNF-κB	Phosphorylated nuclear factor kappa B

poly-HEMA	Poly-hydroxyethylmethacrylate
PTEN	Phosphate and tensin homologue protein
PVDF	Polyvinyl difluoride
Resveratrol	*trans*-resveratrol
RIPA	Radioimmunoprecipitation assay
rpm	revs per minute
SEM	Standard error of the mean
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
TBS-T	Tris-buffered saline and Tween20
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction.

1.1 Overview.

Ovarian cancer is the deadliest gynaecological cancer worldwide, with approximately 69% of sufferers dying from the disease (Lengyel, 2010). According to the most recent survey, it is the fifth most common cause of cancer related deaths in New Zealand women (Ministry of Health, 2013). Ovarian cancer can be classified into two broad groups Type I and Type II and it is Type II that is of most concern. Around 75% of all diagnosed ovarian cancers fall into this group, and these are epithelial-derived tumours that develop quickly, are very aggressive and have a very low early stage detection rate (Cho and Shih, 2009). The current treatment options for ovarian cancer are very limited. It is either treated with aggressive surgery, chemotherapy or a combination of the two (Lengyel, 2010). There are many pitfalls of surgery with the most common being incomplete removal of tumorous cells and post-operative complications (Shih and Chi, 2010). Chemotherapy with the platinum compounds carboplatin or cisplatin and with paclitaxel is non-specific and thus extremely toxic to non-cancerous cells. Furthermore, the development of high rates of resistance to these drugs is also problematic in ovarian cancer cells (Lengyel, 2010). Therefore, the search for more appropriate targeted cancer cell therapies with minimal adverse effects is ongoing (Shukla & Singh, 2011).

Many natural food compounds are of interest as potential chemotherapeutic and chemopreventive agents as they appear to have anti-cancer properties and are nontoxic to homeostatic cells. Of these, resveratrol and EGCG are extremely promising as they have been shown to affect multiple signalling pathways to reduce tumour growth in a variety of human cancers (Singh et al., 2011, Athar et al., 2009). However, these compounds are as yet unproven in ovarian cancer early metastasis and the mechanisms for their potential anti-carcinogenic properties in early metastatic ovarian cancer are unclear.

1.2 Cancer.

Cancer is one of the leading causes of disease and death in developed countries. In 2008 it was estimated that 12.7 million new cases of cancer were diagnosed worldwide, and 7.6 million people died from the disease (Ferlay et al., 2010). In 2012, those figures had increased to 14.1 million new cases and 8.2 million deaths (Ferlay et al., 2013), and cancer rates are predicted to continue to rise in the future, it is estimated that 22.2 million new cases annually will be diagnosed by 2030 (Bray et al., 2012). As the prevalence of cancer increases with age, the steady incline of disease incidence worldwide is in part due to a progressively aging population (Bray et al., 2012). Another key contributing factor to the commonness of cancer is its ability to adapt and circumnavigate current available therapies (Ferlay et al., 2010). Therefore, there is great impetus to elucidate the fundamental survival mechanisms of cancer and develop effective treatment options.

Cancer is a class of genetic diseases that are characterised by uncontrolled cell growth. There are over 100 different types of cancers originating in all areas across the human body and each of these can possess their own physiology. There are also many differing subtypes within each type of cancer and to further complicate matters each subtype can also have its own specific physiology. However, there have been certain common characteristics identified amongst cancer cells that they must adopt to enable their growth. Firstly, cancer cells are resistant to programmed cell death, whilst normal cells eventually undergo apoptosis, in cancer cells intrinsic apoptosis signalling never eventuates. Cells also possess replication immortality that is they are not restricted to a limited number of divisions, and have persistent proliferative signalling, which is arguably the most fundamental of all the traits. Cancer cells are able to induce angiogenesis, the formation of new blood vessels, and are capable of invasion and metastasis. Cancers can also evade immune destruction, growth suppressors and deregulate cellular metabolism (Hanahan and Weinberg, 2011). In addition to this, there are also common underlying mechanisms that enable the cells to acquire these characteristics. These are inflammation, genetic instability and tumour microenvironment alterations (Hanahan and Weinberg, 2011).

1.2.1 Ovarian Cancer.

Ovarian cancer is cancer of the female reproductive organs called ovaries. There are two ovaries in the female body located in the lower pelvic region of the peritoneal cavity. They are connected to the uterus by fallopian tubes and are responsible for producing eggs for reproduction and generating the majority of the female hormones. Ovarian cancer stems from cells on the epithelium (or outer surface) of the ovary, or more rarely from the sex-cord stromal cells which produce hormones or germ cells (Fig. 1.1).

The pathogenesis of ovarian cancers is largely unknown, but a current hypothesis is that they remain localised to the ovary during early development. As development continues the cancer cells slough off the ovary into the peritoneal cavity, and this is frequently associated with the formation of ascetic fluid (fluid in the peritoneal cavity). Once floating in the ascetic fluid the cells aggregate to form spheroids or clusters, which then disseminate throughout the abdominal cavity (Fig. 1.2). This mode of dissemination differs from classic patterns of metastasis, and its unique biological behaviour is largely responsible for the insidious nature of ovarian cancer (Naora and Montell, 2005). Thus, the cancer is able to directly metastasize to other organs also located in the peritoneal cavity (Cho and Shih, 2009).

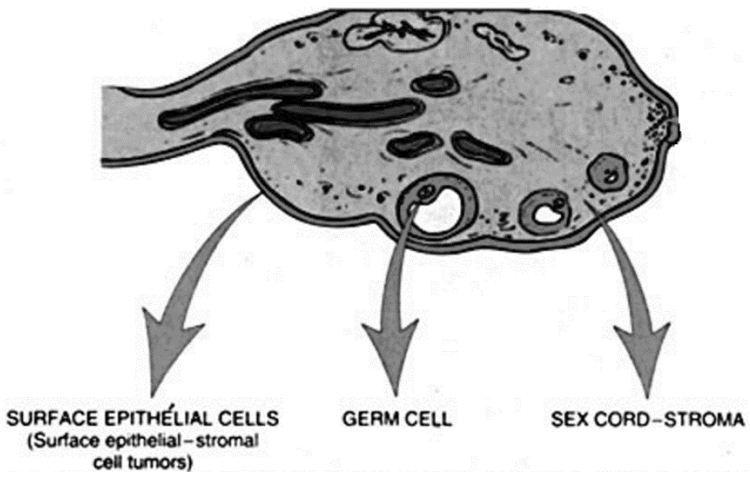

Figure 1.1. The female ovary. Most cases of ovarian cancer arise from epithelial cells on the surface of the ovaries. Germ cells and sex cord-stroma cells give rise to only 10% of ovarian cancer. Image adapted from http://www.pathinformatics.com/smallgroups/reproductive/female/fig5c.htm

The spheroids formed during early development are described as being imperative for free-floating cell survival and growth and believed to significantly hinder the treatment of late stage ovarian cancer (Shield et al., 2009). The origins of ovarian cancer are unknown but a well-accepted theory is that 'incessant ovulation' is primarily the cause. Essentially, it proposes that due to the process of ovulation the epithelium is required to repair itself every month. These epithelial cells, therefore, have to replicate more often which increases the likelihood of genetic damage.

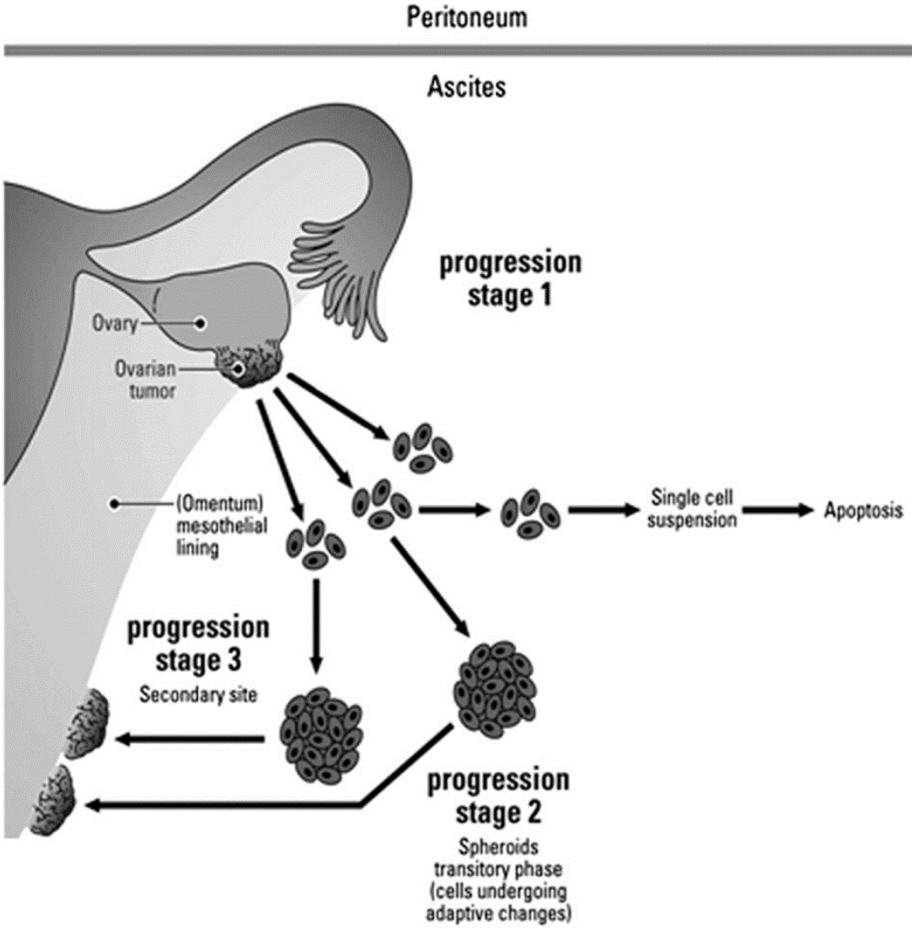

Figure 1.2. Proposed model of early metastasis of ovarian cancer. During progression stage 1 cancerous cells shed off the ovarian tumour into the ascites within the peritoneal cavity. At progression stage 2 the cells aggregate to form spheroids/clusters which are suggested to increase the cancers metastasis. Progression stage 3 is when the spheroids form secondary solid tumours. Image from Shield et al., 2009.

The clinical progression of ovarian cancer is divided into four stages; at stage I the malignancies are restricted to the ovaries, stage II encompasses its localised spread to adjacent pelvic organs and tissues, at stage III the cancer presents on tissues or organs of the upper abdominal cavity and at stage IV the cancer has metastasized to distant extra-peritoneal sites (Fig. 1.3). About 90% of ovarian cancers are epithelial derived carcinomas. These can be further classified as either serous, endometrioid, clear cell or mucinous, based on their morphological appearance (Su et al., 2013). Ovarian cancers are categorized into two broad groups Type I and Type II based on their histology, tumour grade and other general characteristics. The tumour grade describes the proportion of solid tumour growth compared to glandular or papillary structures, also described as the amount of 'differentiation' (Silverberg, 2000). Type I ovarian tumours grow relatively slowly; they are often large and localised to the ovary making them easier to detect. They include serous, endometrioid, mucinous, and some clear cell carcinomas that are of a low grade (1 or 2, i.e. less than 50% solid tumour). Unfortunately, they represent fewer than 25% of aggressive ovarian cancers. Type II ovarian tumours, on the other hand, are mostly grade 3-4 and have almost always metastasized by the time of diagnosis. Accordingly, they are thought to have a quicker development rate than Type I, be much more aggressive and harder to detect at an early stage (Cho and Shih, 2009).

Ovarian cancer has been found to have a higher incidence rate in Caucasian women living in developed countries (Merritt and Cramer, 2010). Occurrence also increases with the onset of menopause and increase in age up to 80 years (Ries et al., 2008). Ovarian cancer also has a high level of familial heredity, with the probability of it developing within a lifetime increasing from less than 2% for an average woman to 7% for women who have at least two family members who had the disease (Schildkraut and Thompson, 1988). The hereditary breast-ovarian cancer *BRCA* mutation also increases the likelihood of ovarian cancer by approximately 30% for carriers (Whittemore et al., 1997), and the estimated population frequency of the mutation is 0.1% (Evans, 2012). A number of other oncogenes have been found to be associated with ovarian cancer besides *BRCA*. Of these, the most prominent include *KRAS*, *BRAF* and *TP53* (Nakayama et al., 2008). The tumour suppressor protein p53, which has a number of cellular roles including control of the cell cycle and stimulation of apoptosis in response to DNA damage. p53 is mutated in 30% of ovarian

cancers, which results in overexpression in about 50% of these cases (Holschneider and Berek, 2000).

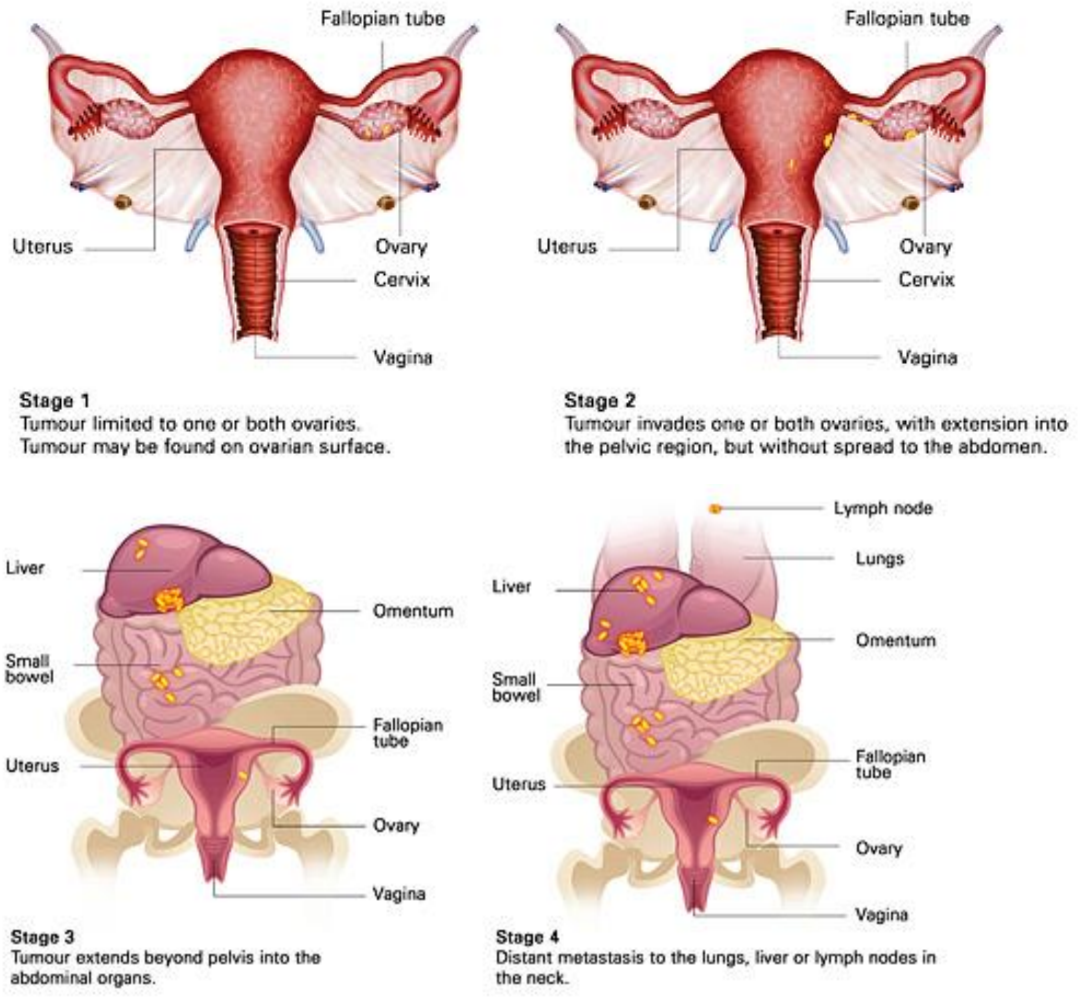

Figure 1.3. The stages of clinical progression of ovarian cancer. Stage 1 –The cancer is limited to the ovaries. Stage 2 –Cancer spreads into the pelvic region. Stage 3 –The cancer metastasizes into the abdominal region. Stage 4 –The cancer reaches distant organs e.g. the lungs or liver.

Image from http://www.kkh.com.sg/HealthPedia/Pages/GynaecologicalCancersOvarian.aspx

Ovarian cancer has a high mortality rate largely due to detection difficulty. Its symptoms are vague and often mistaken for other illnesses, resulting in about 70% of cases only being diagnosed at advanced stages where the survival rate is diminished to just 30% (Cho and Shih 2009). This limits the capacity for successful treatment and more than 50% of women do not survive five years after diagnosis (Coleman et al. 2011).

1.2.2 Current treatment options for Ovarian Cancer.

1.2.2.1 Surgical Management.

Surgery is currently one of the standard treatment options for ovarian cancer. For epithelial ovarian cancers, as a majority are at an advanced stage by the time they are diagnosed, maximal cytoreduction surgery (or debulking) is usually the initial treatment (Shih and Chi, 2010). This form of surgery involves the removal of tumours greater in size than 1cm, complete removal of these tumours is known as optimal cytoreduction. The correlation between debulking and improved outcome was first documented in 1975, where it increased patient survival by over a year (Griffiths, 1975). The theory behind debulking is that larger tumours have poorer blood supply and do not receive optimal doses of chemotherapy and therefore, do not respond to treatment, whereas the smaller tumours left behind should be susceptible to chemotherapy (Shih and Chi, 2010). The size of the tumours left behind is regarded as the strongest prognostic factor for post-operative survival (Aletti et al., 2006). Maximal cytoreduction is limited by the extent of the disease, many surgeons will not remove tumorous sections of the bowl, diaphragm or portal triads of the liver (Aletti et al., 2006), liver parenchymal or lung metastases can also preclude optimal debulking (Shi and Chi, 2010). Debulking is quite a radical surgery and nearly always includes the removal of the ovaries, fallopian tubes, uterus and the lining of the peritoneal cavity known as the omentum (Aletti et al., 2006). This of course renders the women barren and reduces the amount of oestrogen and progesterone produced. This reduction in hormones can lead to early onset of menopause which in turn is accompanied by other complications such as osteoporosis, increased risk of heart disease and other menopausal discomforts.

For the remaining 10% of ovarian cancers that are not epithelial derived surgery is also the first line of treatment. However, due to germ cell and sex cord stromal tumours usually being diagnosed at an early stage, prognosis after surgery is excellent and adjuvant treatment is often not required (Colombo et al., 2009). Furthermore, there is a higher chance that fertility may be retained post-surgery via the removal of only the affected ovary and fallopian tube. This is important as many women affected with these kinds of malignancies are preadolescent or young adults (Colombo et al., 2009). In the rare instances where non-epithelial ovarian cancers present at an advanced stage optimal cytoreduction is undertaken as described above (Colombo et al., 2009).

1.2.2.2 Chemotherapy.

The platinum-based drug cisplatin has been used as a first-line chemotherapy for over 20 years (Burger et al., 2011). Cisplatin is the *cis*-isomer of the diamminedichloroplatinum(II) complex that is, a platinum ion coordinated by two chloride atoms and two ammonia groups with a net charge of +2. The antiproliferative properties of cisplatin were first observed by Rosenberg and colleagues in *Escherichia coli* who then suggested its potential as an anti-cancer drug (Rosenberg et al., 1965). Cisplatin's cytotoxic effects are conferred by its ability to bind directly to DNA (Knox et al., 1986). This interferes with the DNA replication and transcription processes via cross-linking of complimentary DNA strands at adjacent guanine bases (Roberts and Pascoe, 1972). The first evidence of cisplatin effectively reducing ovarian cancer growth was documented in 1974, in what was in fact the first evidence of its antitumor effect on any cancer (Tattersall, 2002). Despite its worldwide use cisplatin has limited efficacy due to negative side-effects and the development of resistance in tumour cells. Its major reported side-effect is nephrotoxicity (Gonzales-Vitale et al., 1977); others are neurotoxicity, ototoxicity, renal toxicity and vomiting (Knox et al., 1986).

Due to the toxicity of cisplatin, other derivatives have been produced and investigated. Of these carboplatin is the only other platinum based compound commonly used worldwide (Lebwohl and Canetta, 1998). Structurally carboplatin is similar to cisplatin however, instead of the chloride groups carboplatin possesses a more stable ligand, the cyclobutane ring, thereby lessening its nephrotoxic potential (Knox et al., 1986). Significant attenuation of ovarian cancer growth was recorded during the first clinical trial of carboplatin (Lebwohl

and Canetta, 1998). The efficacy of carboplatin has been shown to not significantly differ from cisplatin and due to its lower toxicity has become the more preferred chemotherapeutic drug of the two platinum based complexes (Ozols, 1999).
However, due to the quite high occurrence of resistance to platinum based therapy in ovarian cancer researchers began looking at the effectiveness of administering combinations of other chemotherapeutic drugs with a platinum based compound. Paclitaxel was discovered to be active in platinum resistant patients and became a commonly used combination partner (Ozols, 1999). Paclitaxel is a mitotic inhibitor first extracted from bark of *Taxus brevifolia*. It binds to polymerized tubulin, promotes and stabilizes polymerized microtubules thereby arresting cells in mitosis (Cragg and Newman, 2004). To begin with paclitaxel, in conjunction with cisplatin, was the favoured combination for ovarian cancer treatment; today it is paclitaxel and carboplatin that are the more common first line treatment drugs (Tattersall, 2002). However, the toxicity of paclitaxel is also quite excessive. Adverse side effects include peripheral neuropathy, myelosuppression, arthralgias, myalgias and skin problems. Therefore, clinical doses of paclitaxel are limited to achieve antitumor activity, whilst reducing the severity of adverse effects (Cella et al., 2003).
Although the three afore-mentioned chemotherapeutic compounds are the most widely used treatments for epithelial ovarian carcinomas they are not the only ones. Other chemotherapy agents include docetaxel, cyclophosphamide, irinotecan and melphalan. For the management of advanced germ cell or stromal tumours a combination of cisplatin, etoposide and bleomycin is the preferred treatment (Colombo et al., 2009). Although surgery followed by adjunct chemotherapy is the cornerstone of ovarian cancer treatment there are two other options available. One is hormone therapy, which utilises drugs that attenuate oestrogen secretion as oestrogen is a recognised inducer of cell proliferation. Hormone therapy is more often used to treat stromal tumours (Simpkins et al., 2013). The other is radiation, where tumours are targeted with beams of radiation which damage the cancers DNA causing cell death, this treatment is mostly utilised on recurring ovarian carcinomas (Brown et al., 2013).
As ovarian cancers have a high mortality rate, first line treatments are extremely harsh and the incidence of recurrence is extremely likely, it is with great impetus that the search for preventative and or more effective treatment methods continues.

1.3 Natural Dietary Polyphenols.

1.3.1 Resveratrol.

Many natural food compounds are of interest as potential chemotherapeutic and chemopreventive agents as they appear to have anti-cancer properties and be nontoxic to normal cells. Such compounds are also readily available due to their accessibility and wide distribution in edible plants (Shukla and Singh, 2011). The polyphenolic phytoalexin resveratrol is one such compound produced by plants as a defence mechanism against fungal attack (Savouret and Quesne, 2002). It is found in foods such as blueberries, peanuts, mulberries and cocoa, however, it is most abundant in the skin of red grapes and its presence in red wine in relatively high concentrations has turned resveratrol into a compound of great interest (Athar et al., 2009). It was initially isolated from the poisonous roots of white hellebore in 1940 and in 1963 from the roots of a centuries old traditional Asian medicinal plant *Polygonum cuspidatum* known as Ko-jo-kon (Baur and Sinclair, 2006). Resveratrol consists of two phenolic rings linked together by a double bond (Fig. 1.4) this enables it to exist as two diastereomeres, however it is *trans*-resveratrol that occurs more often naturally (Kraft et al., 2009).

There are a wide range of beneficial health effects attributed to resveratrol, including prevention of diabetes and Alzheimer's, inhibition of inflammation and oxidative stress, and cardioprotective effects (Baur and Sinclair, 2006, Athar et al., 2009, Kraft et al., 2009). However, in the late 90's, it was discovered that resveratrol was capable of therapeutic activity against cancer (Jang et al., 1997). Since then, there have been copious studies relating to resveratrol and cancer treatment. Resveratrol's anti-cancer properties have been shown in various human cancers such as skin cancer, lung cancer, liver cancer, breast and prostate cancers (Aggarwal et al., 2004, Athar et al., 2009, Bishayee and Dhir, 2009, Trapp et al., 2010). Resveratrol has been demonstrated to have anti-carcinogenic activity in all three stages of carcinogenesis, i.e. initiation (by regulating signalling pathways that control cell growth and division), promotion (via inflammation and angiogenic pathways) and progression (regulating metastasis) (Shukla and Singh, 2011). However, a majority of these studies have been conducted on either 2D monolayer human cell cultures or *in vivo* animal models.

1.3.2 Acetyl resveratrol.

Analogues of resveratrol have also been subjects of chemopreventive and chemotherapeutic studies as many appear to possess the same characteristics as resveratrol whilst protecting the hydroxyl groups from oxygenation, it is suggested that this shields the compound from rapid metabolism in the liver and or increases cellular uptake (Colin et al., 2009). Therefore, it is possible that these derivatives may act as a sink from which resveratrol can be released as it has been noted that *in vivo* concentrations of other resveratrol analogues can be as much as ten times more than the native compound (Baur and Sinclair, 2006). In acetyl resveratrol (Fig. 1.5), the hydroxyl groups are acetylated thus producing a more stable compound and hydrophobicity is also increased. Therefore, it should be able to cross the cellular membrane more readily than resveratrol (Liang et al., 2013).

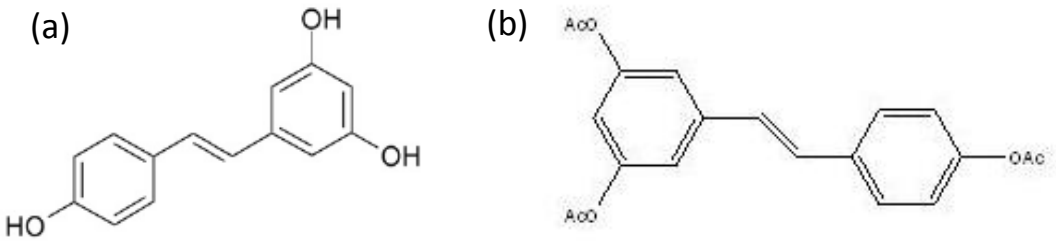

Figure 1.4. The chemical structures of (a) the *trans*-isomer of resveratrol. Image from Kraft et al., 2009. (b) the *trans*-isomer of acetyl resveratrol (3,5,4'-tri-*O*-acetylresveratrol). Image from Liang et al., 2013.

1.3.3 (-)-Epicatechin-3-gallate (EGCG).

The dried leaves of *Camellia sinensis* are used to brew the second most consumed beverage in the world, tea. Tea is mostly available in three types black, green and oolong, green tea roughly accounts for 20% of tea consumed worldwide (Khan and Mukhtar, 2008). Green tea has long been known to be beneficial to the health of humans; it has shown to be beneficial in the treatment of Alzheimer's, diabetes, stroke and obesity (Singh et al., 2011). These benefits have been attributed to green tea's polyphenolic compounds which make up circa 30% of its dried weight. In particular it is the flavanols (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin, (-)epicatechin-3-gallate, (-)-epicatechin, (+)-gallocatechin and (+)-catechin that are most active (Ahn et al., 2003). The most prominent flavanol, in constituency and activity, is EGCG (Kim et al., 2007). EGCG (Fig. 1.6) has also been shown to possess anticarcinogenic properties and has modulated tumour growth and/or spread in breast, skin, colon, lung and liver cancers to name a few (Lu et al., 2002, Ahn et al., 2003, McLoughlin et al., 2004, Singh et al., 2011, Zhang et al., 2012). Like resveratrol, it has also been demonstrated that EGCG is capable of affecting multiple signalling pathways to effect cancer inhibition (Singh et al., 2011). However, there are few studies on the effects of EGCG on ovarian cancer and to the best of my knowledge there have been none conducted in a 3D model.

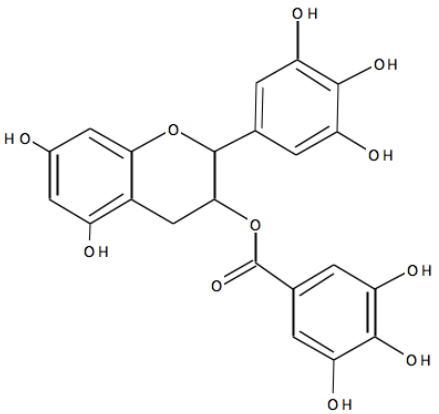

Figure 1.5. The chemical structure of (-)-Epicatechin-3-gallate. Image from Khan and Mukhtar, 2008.

1.4 Vascular Endothelial Growth Factor (VEGF) and The Nuclear Factor kappa B (NF-κB) Signalling Pathway.

VEGF is a glycoprotein that is significantly involved in angiogenesis. Angiogenesis is the physiological process of developing new blood vessels from already existing ones. This process is thought to be vital to the growth and survival of any malignant tumour (Shimizu et al., 2010). Overexpression of VEGF has been observed in ovarian cancer and is associated with poor prognosis for ovarian cancer sufferers (Yu et al., 2013). There is a strong possibility that VEGF is important for the metastasis of ovarian cancer and it has been shown that resveratrol and EGCG are capable of downregulating VEGF secretion (Spinella et al, 2006, Dann et al., 2009).

Signalling pathways that control cell proliferation, survival and angiogenesis are of interest for the development of targeted therapy (Rayet and Gelinas, 1999). The NF-κB pathway appears to be quite prominent in the angiogenesis process and the growth of cancer cells (Athar et al., 2009). The NF-κB protein itself has been identified as a target of resveratrol and EGCG in other types of cancer (Ahmad and Mukhtar, 1999, Athar et al., 2009). Therefore, the NF-κB signalling pathway is a strong candidate for being the co-ordinator of VEGF secretion in metastatic ovarian cancer.

1.5 Three-Dimensional *in vitro* model.

Since its development in 1907, two-dimensional (2D) cell culturing has been one of the cornerstones of research in cellular biology. 2D cell cultures are grown as a monolayer adhered to the bottom of a vessel with a medium for nutrition and are kept at body temperature (Breslin and O'Driscoll, 2013). However, of late the correlation of 2D culture results and *in vivo* situations has been called into question and other experimental setups such as three-dimensional (3D) cell cultures have begun to supersede the 2D model. It has been shown that 2D cultures can be significantly divergent in their cell-cell and cell-matrix interactions, morphology, receptor and oncogene expression, and differentiation from cultures grown in a more physiologically relevant 3D environment (Yamada and Cukierman, 2007). In normal epithelial cells organization, signalling and protein secretion *in vivo* is more similar to the 3D approaches than the 2D settings (Yamada and Cukierman,

2007). With regards to cancer biology, a distinct advantage 3D models have over 2D is the aggregates/spheroids produced are composed of cells with differing phenotypes, there are proliferating, non-proliferating and necrotic cells, this more closely resembles in vivo human tumours (Kim, 2005). 2D and 3D cultures also display drug response variances, for example when treated with a classical chemotherapeutic drugs 5-fluorouracil and tirapazamine cell viability was only 5% in the 2D culture whereas viability was up at 75% in the 3D model (Breslin and O'Driscoll, 2013).

There are several different methods available when setting up 3D experiments; these include hanging drop, matrices and scaffolds, and microfluidic cell culture platforms (Breslin and O'Driscoll, 2013). However, the forced floating method was deemed the most appropriate for this investigation. This system was developed by Ivascu and Kubbies in 2006 (Breslin and O'Driscoll, 2013) it is relatively simple to execute and highly reproducible. Plates are coated with a polymer to prevent cell attachment this promotes cell-cell interactions, thus, cells aggregate and form clusters/spheroids. This method was chosen for a variety of reasons. Firstly, the spheroids/clusters are easily accessible, in other systems that involve matrices or scaffolds it can be difficult to extract the aggregates formed (Breslin and O'Driscoll, 2013). It is compatible with investigations into drug efficacy, protein expression and identifying potential individualised cancer therapies (Yamada and Cukierman, 2007). Most importantly it mimics the microenvironment and cell formation of ovarian cancer aggregates in ascetic fluid much more closely than 2D models and other 3D models. Cell shape and environment have been recognised as important determinants of gene expression and behaviour of the cell (Kim, 2005).

1.6 Hypothesis and Aims.

Although the anti-carcinogenic properties of the natural dietaries resveratrol and EGCG have been extensively studied there is a lack of information regarding their effects on cell spheroids/clusters of ovarian cancer. I hypothesise that resveratrol, acetyl resveratrol and EGCG will exhibit anti-tumour activities in cell spheroids/clusters of ovarian cancer cells, and that these anti-tumour activities are mediated by NF-κB attenuation.

1.6.1 Aims.

To study the effects of resveratrol, acetyl resveratrol and EGCG on cellular growth and metabolism in metastatic ovarian cancer.

To study the combination effect of EGCG and resveratrol in metastatic ovarian cancer.

To study the effect resveratrol, acetyl resveratrol and EGCG have on the secretion of VEGF and IL-8.

To study the effect resveratrol, acetyl resveratrol and EGCG have on the activation of the NF-κB pathway.

1.6.2 Model organisms.

In this study, two immortal ovarian cancer cell lines were utilised SKOV-3 and OVCAR-5. These cell lines were chosen as it had previously been demonstrated that when grown in the 3D model the spheroids/clusters are morphologically quite different. The SKOV-3 spheroids/clusters grow large enough to see with the naked eye and are very irregular in shape, whereas the OVCAR-5 spheroids/clusters are much smaller and much more uniform in shape.

Chapter 2.

Materials and Methods.

2.1 Materials.

2.1.1 General solutions, buffers and media.

2.1.1.1 10x Phosphate Buffer Solution (PBS).

10x PBS was made up using the following, 1.4% Na_2HPO_4, NaH_2PO_4 for pH adjustment to 7.4, 9% NaCl, 0.2% KCl and dissolved in MilliQ H_2O up to 500ml.

2.1.1.2 10x Non-sterile Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA).

10x Trypsin-EDTA was made up using the following, 2.5% Trypsin powder, 0.75% EDTA, dissolved in 200ml 1x PBS. pH adjusted to 8 by adding NaOH.

2.1.1.3 Tris(4-(dimethylamino)phenyl)methylium chloride (Crystal Violet).

Crystal violet was made up using the following, 0.2% crystal violet powder in 100ml 2% ethanol.

2.1.1.4 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 working media (DMEM-F12).

DMEM-F12 base media was made up using the following, 1 packet of Alpha medium and 0.37% $NaHCO_3$ in 1l MilliQ H_2O. pH was the adjusted to between 7.1 and 7.3 with NaOH. This was then filtered in a cell culture laminar flow cabinet.

This base media was supplemented with 5% (v/v) FBS, PenStrep antibiotics at a concentration of 100units/ml penicillin and 100µg/ml streptomycin, 2mM glutaMAX™ and 2µg/ml Fungizone®.

2.1.1.5 Minimum Essential Medium working media (MEM).

MEM base media was made up using the following, 1 packet of Alpha medium and 0.22% $NaHCO_3$ in 1l MilliQ H_2O. pH was the adjusted to between 7.1 and 7.3 with NaOH. This was then filtered in a cell culture laminar flow cabinet.

This base media was supplemented with 5% (v/v) FBS, PenStrep antibiotics at a concentration of 100units/ml penicillin and 100µg/ml streptomycin, 2mM glutaMAX™ and 2µg/ml Fungizone®.

2.1.1.6 Tris-buffered Saline Solution (TBS).

TBS was made up containing the following, 10ml 2M Tris-HCl (pH 7.5), 0.82% NaCl and 1l MilliQ H_2O.

TBS-T was made by adding 0.1% (v/v) Tween-20 to TBS.

2.1.1.7 5x Sample Buffer.

5x sample buffer was made up by using the following, 0.6ml 1M Tris-HCl pH 6.8, 5ml 50% glycerol, 2ml 10% SDS, 2ml 1% bromophenol blue and 0.4ml d.H_2O.

2.1.1.8 Radio-ImmunoPrecipitation Assay (RIPA) Lysis Buffer.

RIPA lysis buffer was made up using the following, 20 pierce protease inhibitor cocktail tablets dissolved in 250ml RIPA buffer.

2.1.1.9 Blocking buffer for immunofluorescence.

Blocking buffer was made up containing the following, 4% BSA in PBS.

2.1.2 Potential Anti-carcinogenic Drugs.

2.1.2.1 Resveratrol.

Resveratrol was made up containing the following, 2mM resveratrol in 50% (v/v) DMSO and 50% (v/v) PBS.

2.1.2.2 Acetyl Resveratrol.

Acetyl Resveratrol was made up containing the following, 2mM Acetyl-resveratrol in 50% (v/v) DMSO and 50% (v/v) PBS.

2.1.2.3 Epigallocatechin-3-gallate (EGCG).

EGCG was made up containing the following, 1mM Epigallocatechin-3-gallate in working media (either DMEM-F12 or MEM).

2.1.2.4 NF-kB inhibitor.

NF-kB inhibitor was made up containing the following, 2mM Bay 11-7085 in 100% DMSO.

2.1.3 Enzyme-Linked ImmunoSorbent Assay (ELISA) solutions and buffers.

2.1.3.1 Washing Buffer.

Wash buffer was made up containing the following, 0.05% Tween-20 in 1x PBS.

2.1.3.2 VEGF Reagent Diluent.

VEGF reagent diluent was made up containing the following, 1% BSA in 1x PBS.

2.1.3.3 IL-8 Reagent Diluent.

IL-8 reagent diluent was made up containing the following, 0.1% BSA, and 0.05% Tween-20 in TBS.

2.1.3.4 IL-8 Blocking Buffer.

IL-8 blocking buffer was made up containing the following, 1% BSA, 0.05%NaN_3 in 1x PBS.

2.1.4 Western Blot Solutions and Buffers.

2.1.4.1 2M Tris-HCl (pH 8.8).

2M Tris-HCl was made up containing the following, 24.2% Tris base, concentrated HCl to pH and MilliQ H_2O to make total volume 100ml.

2.1.4.2 1M Tris-HCl (pH 6.8).

1M Tris-HCl was made up containing the following, 12.1% Tris base, concentrated HCl to pH and MilliQ H_2O to make total volume 100ml.

2.1.4.3 Solution B.

Solution B was made up using the following, 75ml 2M Tris-HCl (pH 8.8), 4ml 10% SDS in d.H_2O and 21ml MilliQ H_2O.

2.1.4.4 Solution C.

Solution C was made up using the following, 50ml 1M Tris-HCl (pH 6.8). 4ml 10% SDS in d.H_2O and 46ml MilliQ H_2O.

2.1.4.5 SDS-PAGE Separating Gel (10%).

SDS-PAGE separating gel was made up using the following, 2.8ml d.H_2O, 1.25ml solution B, 1.66ml solution A (30% (w/v) acrylamide), 25µl 10% (w/v) $(NH_4)_2S_2O_8$ and 2.5µl TEMED.

2.1.4.6 SDS-PAGE Stacking Gel (7%).

SDS-PAGE stacking gel was made up using the following, 1ml d.H_2O, 0.5ml solution C, 0.5ml solution A (30% w/v) acrylamide), 20µl 10% (w/v) $(NH_4)_2S_2O_8$ and 2.5µl TEMED.

2.1.4.7 Electrophoresis Buffer.

Electrophoresis buffer was made up containing the following, 0.3% Tris, 1.4% glycine, 0.1% SDS and MilliQ H_2O to make 1l.

2.1.4.8 Transfer Buffer.

Transfer buffer was made up using the following, 0.19% Tris, 0.9% glycine dissolved in 2l MilliQ H_2O.

2.1.4.9 Blocking Solutions.

Blocking solutions were made up containing the following, either 5% (w/v) skim milk or 2% (w/v) BSA in TBS-T.

2.1.4.10 Antibody Solutions.

Antibody solutions were made up containing the following, either 2.5% (w/v) skim milk or 1% (w/v) BSA in TBS-T.

2.2 Methods.

2.2.1 Cell Cultures.

The well-established human cancer cell lines ovarian adenocarcinoma OVCAR-5 and SKOV-3, were kindly provided by co-supervisor Dr. Chitcholtan (Department of Obstetrics and Gynaecology, University of Otago, Christchurch). OVCAR-5 was maintained in DMEM-F12 working media and SKOV-3 was maintained in MEM working media.

Both cell lines were maintained continuously in 50ml culture flasks containing the relevant working media in a humidified 5% CO_2 incubator at 37°C. All subculturing procedures and experimental set ups were carried out under sterile conditions in a cell culture laminar flow cabinet. The media was replaced with fresh working media every two days until the cells were almost confluent, as assessed using a light microscope. At this stage, cells were sub-cultured. To sub-culture, the media was discarded, the flask was then rinsed with sterile 1x

PBS and 5ml of sterile 1x Trypsin-EDTA was added. Flasks were then incubated for 15-25 minutes until most cells no longer adhered to the bottom of the flask. Cells were then transferred into a 15ml tube and centrifuged at 1500rpm for 5 minutes, after which the supernatant was discarded. The cell pellet was then resuspended in the appropriate working media. A sample of the cell suspension was then taken and diluted in half with 1x PBS and a cell count was made using a haemocytometer. Approximately 300µl of the cell suspension was added to a new culture flask with fresh working media for cell line maintenance.

2.2.1.1 Experimental 3D Spheroid/Clusters Cell Cultures.

The following procedure was performed prior to sub-culturing cells in 12 well plates for each experiment. 0.5ml of 2.4% (w/v) poly-HEMA dissolved in 95% ethanol at 70°C was added to each well, the plates were then transferred to an orbital shaker set at 50rpm at a temperature of 37°C and left overnight with the lid slightly ajar to enable drying and setting of the polyHEMA. The plates were then removed and left at room temperature for 24 hours to harden, the coated plates were then ready to use. Before adding cells the coated plates were washed with 1x PBS.

For each experiment the average cell count that was obtained during sub-culturing was used to calculate the volume of cell suspension required for $1x10^5$ cells per well. This volume was then added to each well in the coated 12 well plates and relevant working media was added to make a total volume of 1ml per well. The spheroids were grown for 6 days before treatment and media in each well was replaced with fresh working media every two days.

2.2.1.2 Drug Treatment.

After 6 days of culturing, the spheroids/clusters were singly treated with resveratrol, acetyl resveratrol, EGCG or NF-kB inhibitor at one of three concentrations (10µM, 20µM and 30µM). For the control, DMSO was added at a volume equal to that of the drug used in the highest concentration treatment. For the combination experiments 10µM of resveratrol and 10µM EGCG were combined and 20µM of each used singly. In each experiment, there were three replicate wells for each treatment. Plates were kept in the incubator and

spheroids were treated for either 2, 4 or 6 days, with fresh media and drugs administered every 2 days.

2.2.2 Alamar Blue Assay.

Cellular metabolism was assessed after drug treatment by the addition of Alamar Blue. Alamar Blue (resazurin) is a colourimetric REDOX indicator that is irreversibly reduced to the pink coloured compound resorufin in the presence of respiring cells. 0.5ml of the media was removed and discarded from each well before 50µl of Alamar Blue was added to each well. Plates were then incubated at 37°C for 4 hours before a sample was taken from each well and loaded onto a 96 well plate. The colour change was measured by absorbance at 570nm and 600nm using a microplate reader (SpectraMax® M5, Molecular Devices). The difference between the absorbances was used as a measure of metabolic activity.

2.2.3 Crystal Violet Assay.

Following the Alamar Blue Assay, cell numbers were indirectly determined by staining cellular DNA purple with crystal violet. The light absorbance of crystal violet at a wavelength of 570nm enables quantification of the amount of DNA in a sample which is taken as being proportional to the total cell number. 1ml 1x PBS was added to each well to rinse out the Alamar Blue. Spheroids were then transferred to 5ml tubes, 3ml 1x PBS added and then centrifuged at 2500rpm for 5 minutes. The supernatant was discarded and 300µl of 10x non sterile trypsin-EDTA was added to separate the cells. After incubation for 30 minutes at 37°C, cells were pelleted by centrifugation at 2500rpm for 5 minutes and the supernatant discarded. 50µl of crystal violet was added and incubated at room temperature for 15 minutes. The cells were then washed with MiliQ H_2O to remove any non DNA-bound dye and centrifuged at 3500rpm. The wash was repeated three times, then the cells were lysed with 2% (w/v) SDS in distilled H_2O. The cell solutions were then loaded onto a 96-well plate and the optical density at 560nm measured using the microplate reader.

2.2.4 VEGF and IL-8 ELISA.

Treatment media of the spheroids/clusters were collected and centrifuged at 3000rpm for 5 minutes. The supernatant was aspirated, and stored at -20°C for the analysis of VEGF and IL-8 secretion. For the sandwich ELISAs, the protocol from the manufacturer was strictly adhered to and is as follows. Capture antibody diluted in 1x PBS was added to each well being used (as calculated previously) in a 96-well ELISA plate (Nunc, Thermo scientific, Illinois, USA) and incubated at room temperature overnight. The wells were then washed four times using the wash buffer and blocking buffer (Reagent diluent for VEGF) was added. After incubation at room temperature for 1 hour the wells were again washed four times, then two sets of standards and 2x diluted (with 1x PBS) samples were added. These were incubated at room temperature for two hours. The plate was washed four times then detection antibody diluted with reagent diluent was added and incubated for another two hours at room temperature. After washing four times streptavidin-HRP diluted with reagent diluent was added and the plate was then incubated at room temperature in the dark for 20 minutes. The wells were again washed four times and ultra TMB-ELISA added. After 7-10 minutes $2NH_2SO_4$ was added and the absorbance at 450nm was measured using the microplate reader.

2.2.5 BCA Protein Assay.

BCA protein assays were carried out using an assay kit (Thermo Scientific, Illinois, USA) in order to determine the total protein in the pellets. This was conducted to normalise the protein concentrations in each sample for ELISA and Western Blotting. Pellets were lysed by adding 200µl of ice-cold RIPA lysis buffer to each sample and incubating for 30 minutes on ice. 25µl of each unknown sample was then loaded onto a 96-well plate along with two sets of standards. To each well 200µl of the working reagent (50:1, Reagent A:B) was added, the contents of the wells on the plate were mixed on an orbital shaker then incubated at 37°C for 30 minutes. Absorbance was measured at 562nm using the microplate reader.

2.2.6 Western Blotting.

After the BCA protein assay 20µl of 5x sample buffer was added to each sample. The samples were vortexed, heated at 99°C for 10 minutes, put on ice for 10minutes followed by centrifugation for 5 minutes at 10,000rpm. Gels were prepared with the 10% SDS-PAGE separating gel and the 7% stacking gel. Samples were loaded onto gels at a volume, determined by the total protein concentration, required to load 10µg of protein per sample. MagicMark™ Western Protein Standard and Precision Plus Protein™ standard were used as SDS-PAGE markers. The SDS-PAGE was run for approximately 120 minutes at 120 volts using Electrophoresis buffer. Following SDS-PAGE the separated proteins were transferred onto polyvinyl difluoride (PVDF) membranes (BIO-RAD, Hercules, USA), running at 100 volts for 60 minutes in cold Transfer buffer. Membranes were then put in the appropriate blocking solution for 60 minutes. The blocking solution was discarded, membranes washed with MilliQ water for 5 minutes, then diluted primary antibody added and incubated overnight at 4°C. The primary antibody was then discarded and membranes were washed for 10 minutes with TBS-T on an orbital shaker. This wash step was repeated four times with fresh TBS-T each time before incubation for 90 minutes at room temperature with the appropriate diluted secondary antibody. The membranes were again washed four times with TBS-T and then developed using a chemiluminescence detection kit (Amersham™ ECL™ Prime Western Blotting Detection Reagent Kit, GE Healthcare). Protein bands were imaged and densitometry analysis performed using Alliance 4.7, Uvitec software (Cambridge, UK).

2.2.7 Frozen Sectioning and Immunofluorescence.

2.2.7.1 Sample preparation.

Spheroids were harvested in 1.5ml eppendorf tubes after growing and treating as described above. After centrifugation at 2000rpm for 5 minutes media was removed and discarded. 1ml of an ice cold 50/50 methanol acetone solution was added to each tube and tubes were kept at -20°C.

2.2.7.1.1 SKOV-3

Samples were centrifuged at 2500rpm for 5 minutes and the supernatant removed and discarded. Pellets were then washed with 1ml of PBS, centrifuged at 2500rpm and the supernatant discarded. This wash step was repeated twice, before 200µl of aniline blue was added to each tube and incubated at room temperature for 20 minutes. Tubes were again centrifuged at 2500rpm, the supernatant was discarded and pellets were washed as described above a further three times. Samples were then prepared for sectioning as the spheroids of SKOV-3 are too thick to visualise with the microscope without sectioning. Approximately 0.5ml CryO-Z-T was added to each tube and pre-labelled plastic rectangle plates evenly covered with CryO-Z-T. The stained spheroids with the CryO-Z-T were then pipetted onto the covered plate and any large bubbles that formed were removed. Plates were frozen overnight at -80°C and then stored at -20°C.

2.2.7.1.2 OVCAR-5

Samples were centrifuged at 2500rpm for 5 minutes and the methanol/acetone supernatant discarded. The pellet was then washed with 1ml of 1x PBS, centrifuged at 2500rpm for another 5 minutes and the supernatant again discarded. The wash was repeated once more and then 200ml of 1x PBS added to each sample. No sectioning was required for OVCAR-5 as this cell line formed small clusters that were easily visualised under the epifluorescent microscope.

2.2.7.2 Slide preparation.

2.2.7.2.1 SKOV-3.

Superfrost plus microscope slides (Menzel-glaser, Germany) were first appropriately labelled in pencil. A chuck and block were placed in dry ice to freeze. CryO-Z-T was then utilised to mount the frozen sample onto the chuck. Samples were kept on dry ice when not in use. The mounted sample was loaded into a cryostat and sectioned. Two slices 7µM thick were placed on each slide and six slices per sample were collected. Slides were stored at -20°C.

2.2.7.2.2 OVCAR-5.

Firstly Polysine microscope slides (Thermofisher, NZ) were labelled in pencil and a 20mm silicone isolator (Invitrogen, NZ) adhered. The 200ml samples were then loaded onto the appropriate slide and left to dry at 37°C. Once dry the isolator was removed.

2.2.7.3 Sample Staining.

SKOV-3 and OVCAR-5.

A super pap pen was utilised to enclose each sample. 200µl of blocking buffer was then added to each sample and incubated at room temperature for 1 hour. The slides were then washed for 10 minutes with PBS before primary antibodies were administered at a dilution factor of 1:200 in PBS with 2% BSA. These were incubated overnight at 4°C.

Slides were then washed twice for 10 minutes with PBS and secondary antibody (goat anti-rabbit IgG conjugated with Atto 594nm) added at a 1:1000 dilution with 2% BSA in PBS. After one hour incubation at 37°C, the samples were then stained with 10µg/ml Hoechst stain for 20 minutes. The slides were then washed three times with PBS + 0.1% Tween-20, 2-3 drops anti-fading solution (2g/l p-phenylenediamine in 80% glycerol) applied and covered with a coverslip. Slides were observed and imaged with an epifluorescence microscope (AxioVision 4.5. Apotome software, Carl Zeiss, Oberkochen, Germany).

2.2.8 Statistical Analysis.

Statistical analyses were performed using GraphPad Prism® software (La Jolla, CA, USA). Experimental results were assessed for significance using an unpaired t-test (using $P < 0.05$ as a measure of statistical significance).

Chapter 3.

Effects of Dietary Polyphenols on Cellular Metabolism and Growth of Ovarian Spheroids/Clusters.

3.1 Introduction.

3.1.1 Cell Division in Normal and Cancerous Cells.

Cell division is how all somatic animal cells including cancer proliferate. The cell cycle begins when the cell is produced from the mother cell and lasts until the cell undergoes its own division into two daughter cells. The cell cycle can be divided into two stages, interphase and mitosis. A majority of the cell cycle is interphase, this is a period for DNA replication and growth, which can be further divided into 3 phases, G_1 (first growth phase), S (synthesis) and G_2 (second growth phase). DNA is replicated during S phase whilst proteins and other cellular components are produced during the other two phases (Alberts et al., 2007). The mitotic phase is the stage during which the cell divides into two identical daughter cells and accounts for about 20% of the cell cycle. In general the nuclear envelope breaks down, the replicated chromosomes are separated and the two identical sets of chromosomes are enveloped by their own nucleus. The cell then divides into two daughter cells containing roughly equal shares of organelles and cytoplasm (Alberts et al., 2007). Normal cells will only undergo mitotic division a set number of times before ceasing being able to reproduce this is known as the hayflick limit (Shay and Wright, 2000). Cancer cells however, do not become senescent and are able to divide indefinitely. In normal cells, the cell cycle is tightly regulated by cell cycle regulators such as cyclins, cyclin-dependent kinases (CDKs) and checkpoint proteins. Normal cells respond to extracellular signals

through the regulators at G_1 phase and exit from the cell cycle, entering G_0 (resting) phase, cells in G_0 make up a major part of the human body's non-cycling cells (Vermeulen et al., 2003). In contrast, cancer cells have mutations usually in either proto-oncogenes which are often involved in signal transduction of mitogenic signals, or tumour suppressor genes. Inactivation of these suppressor genes disrupts proteins that are usually responsible for inhibiting cell cycle progression. Thus, cancer cells are unable to exit the cell cycle and continuously proliferate (Sherr, 1996).

3.1.2 Apoptosis in normal and cancerous cells.

In animal tissue homeostasis is maintained through the eradication of damaged or superfluous cells by cell death or apoptosis. Apoptosis morphology is characterized by chromatin condensation, cytoplasmic shrinkage, nuclear fragmentation, membrane blebbing, DNA fragmentation, and the formation of apoptotic bodies within the cytoplasmic content. During apoptosis, caspases which are cysteine-aspartic proteases, cleave vital cellular substrates and are essential to programmed cell death (Lowe and Lin, 2000).

The activation of apoptosis is achieved by either extrinsic or intrinsic signals. Extrinsic signals bind to 'death receptors' which trigger their downstream caspases. Intrinsic signals are mediated by the mitochondria and induced by things such as DNA damage or viral infection (Piotrowska et al., 2012). Destabilized mitochondria release cytochrome-c which subsequently causes the oligomerization of the apoptotic protease activating factor 1 (Apaf-1) and the formation of apoptosomes. The apoptosomes then activate caspases and induce proteolysis of the cell (Reimann and Schmitt, 2007). The more common mutations in cancers that enable them to elude cell death occur in the *bcl-2* and *p53* genes (Lowe and Lin, 2000). The contribution of both the intrinsic and extrinsic pathways to cancer cell apoptosis depend on differences between cancer cell types and the cytotoxic agent utilised (Piotrowska et al., 2012).

3.1.3 Natural food compounds and cancerous cell growth.

EGCG was one of the first natural food compounds to be recognised for its anti-proliferative effect on cancers in the late 80's (Ahmad and Mukhtar, 1999). It has been demonstrated that EGCG affects several biological pathways in cancer cell proliferation, for example it supresses aberrant CDKs, cyclins and *Ras* –products of which regulate cell growth (Singh et al., 2011). EGCG has also been shown to be able to upregulate apoptotic caspases and modulate anti-apoptotic proteins such as Bcl-2 and survivin, as well as many others.

Resveratrol's adverse effect on cancer growth was first observed in 1997 (Shukla and Singh, 2011). Since then there have been many studies into the mechanisms of resveratrol's anti-tumorous effects (Baur and Sinclair, 2006, Athar et al., 2009, Cui et al., 2010, Gatouillat et al., 2010). Thus far, it has been revealed that resveratrol is also active in several cell growth and apoptotic pathways in cancer cells and affects the expression of cyclins, PTEN (a tumour suppressor), caspases and CDKs to name a few (Shukla and Singh, 2011). Unlike EGCG and resveratrol the effects of acetyl resveratrol on cancer appears to have not yet been investigated.

3.1.4 Aims of Chapter 3.

In this chapter the overall aim is to investigate the effect that resveratrol, acetyl resveratrol and EGCG have on the cellular metabolism and growth of ovarian cancer spheroids/clusters utilising the well-established ovarian cell lines SKOV-3 and OVCAR-5.

3.2 Methods and Materials.

Experimental 3D spheroid cell cultures -See Chapter 2 for full method and materials.

Alamar Blue Assay -See Chapter 2 for full method and materials.

Crystal Violet Assay -See Chapter 2 for full method and materials.

3.3 Results.

3.3.1 Two Day Treatment Effects on Cellular Metabolism and Proliferation.

For resveratrol there was no change in cellular metabolism in both the OVCAR-5 and SKOV-3 cell lines after 2 days of treatment (Fig 3.1 a, b). Similar results were also observed in acetyl resveratrol treated cells (Fig. 3.2 a, b). However, after two days of treatment with 30µM EGCG (Fig. 3.3 b) OVCAR-5 responded with a small increase and there is also a non-significant increase for the same cell line in the combination experiments (Fig. 3.3 b).

Cell growth for SKOV-3 was decreased after 2 days treatment with 20µM and 30µM resveratrol (Fig. 3.1 c) by 13% and 19%, respectively. The acetyl resveratrol and EGCG treatments for 2 days did not affect either cell line (Fig. 3.2 c, d Fig. 3.3 c, d). Unexpectedly, the SKOV-3 cell growth increased by 23% ($p= 0.038$, t-test, $n= 3$) when treated with the combination of resveratrol and EGCG (Fig. 3.4 d).

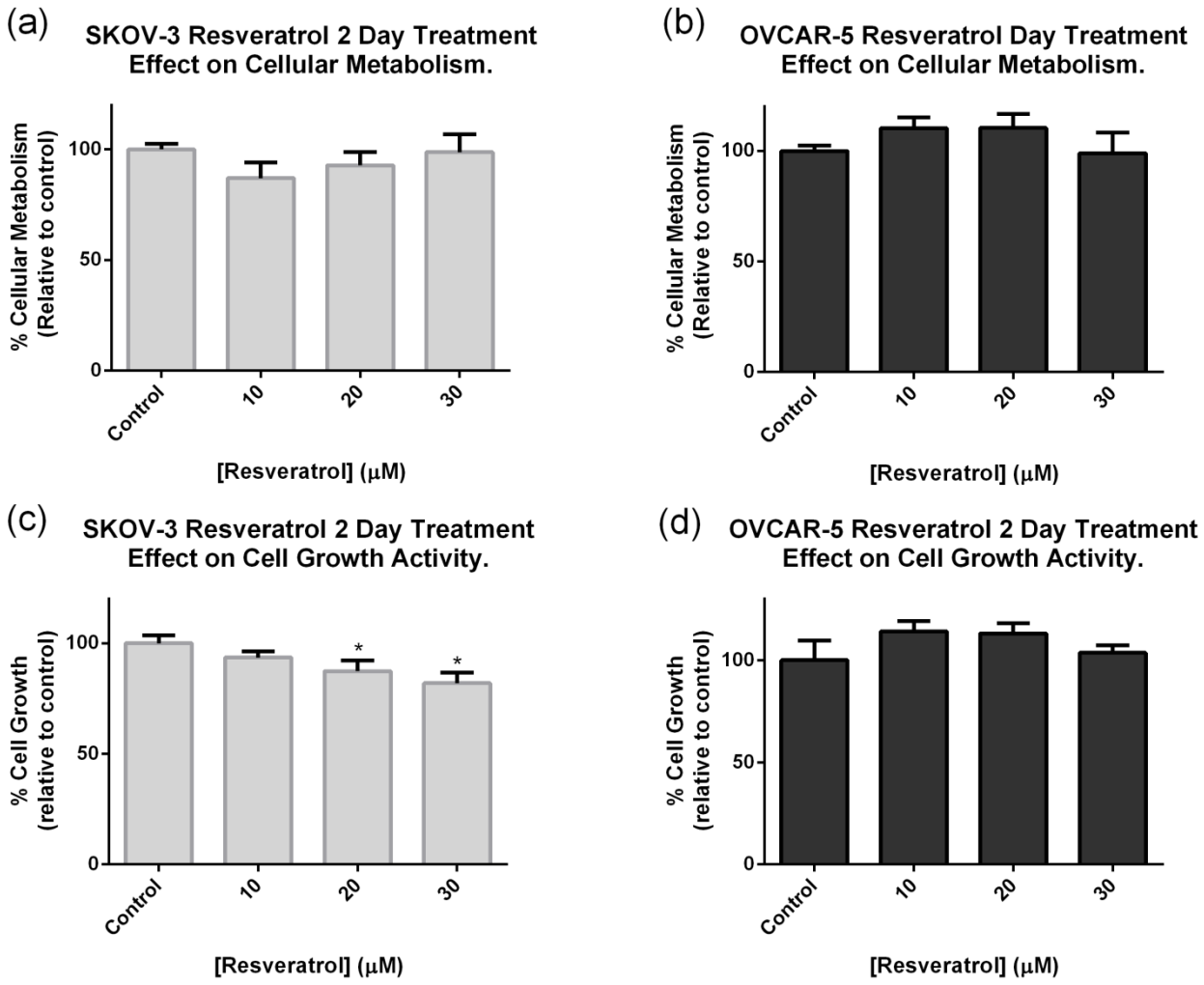

Figure 3.1. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of resveratrol for 2 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

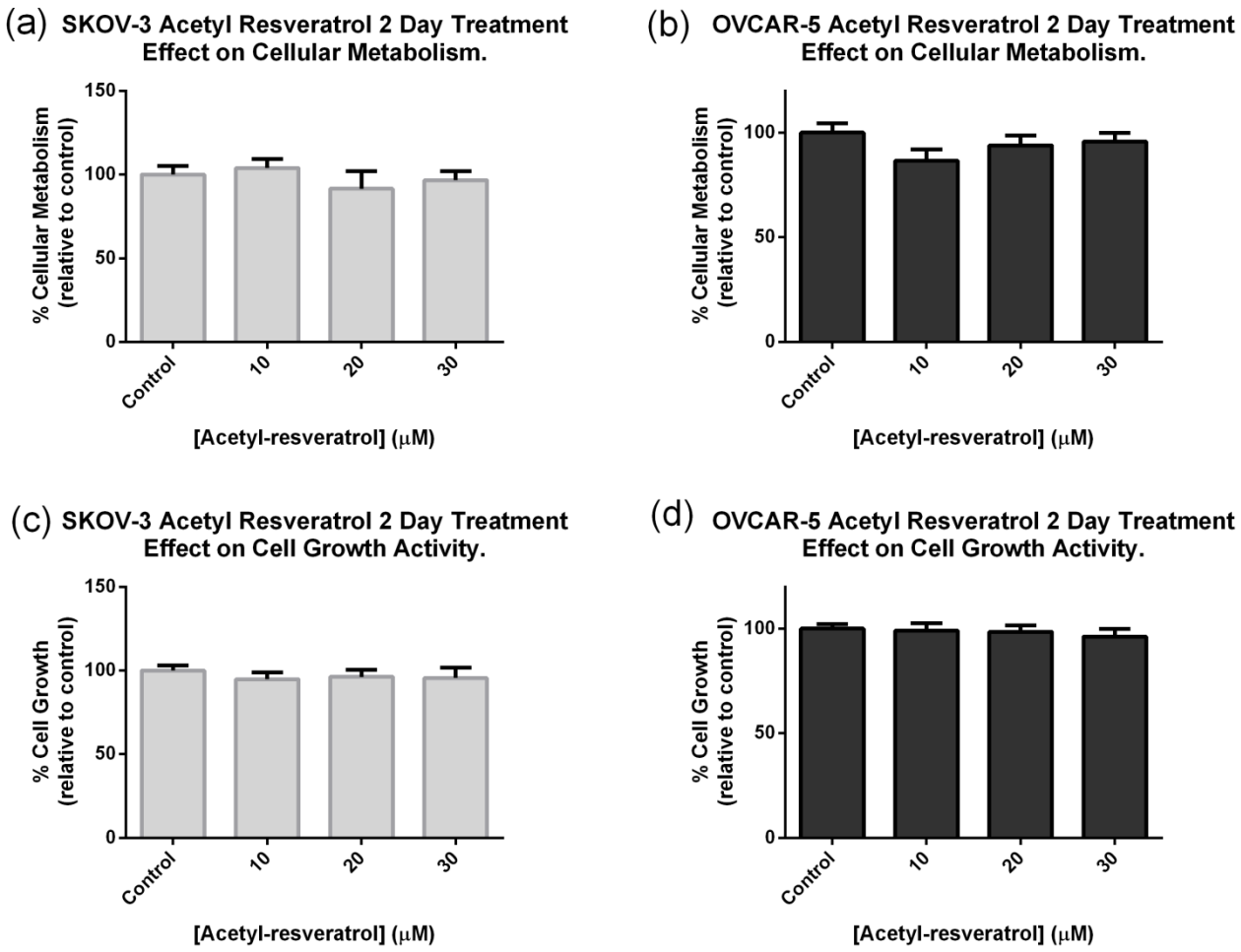

Figure 3.2. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of acetyl resveratrol for 2 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

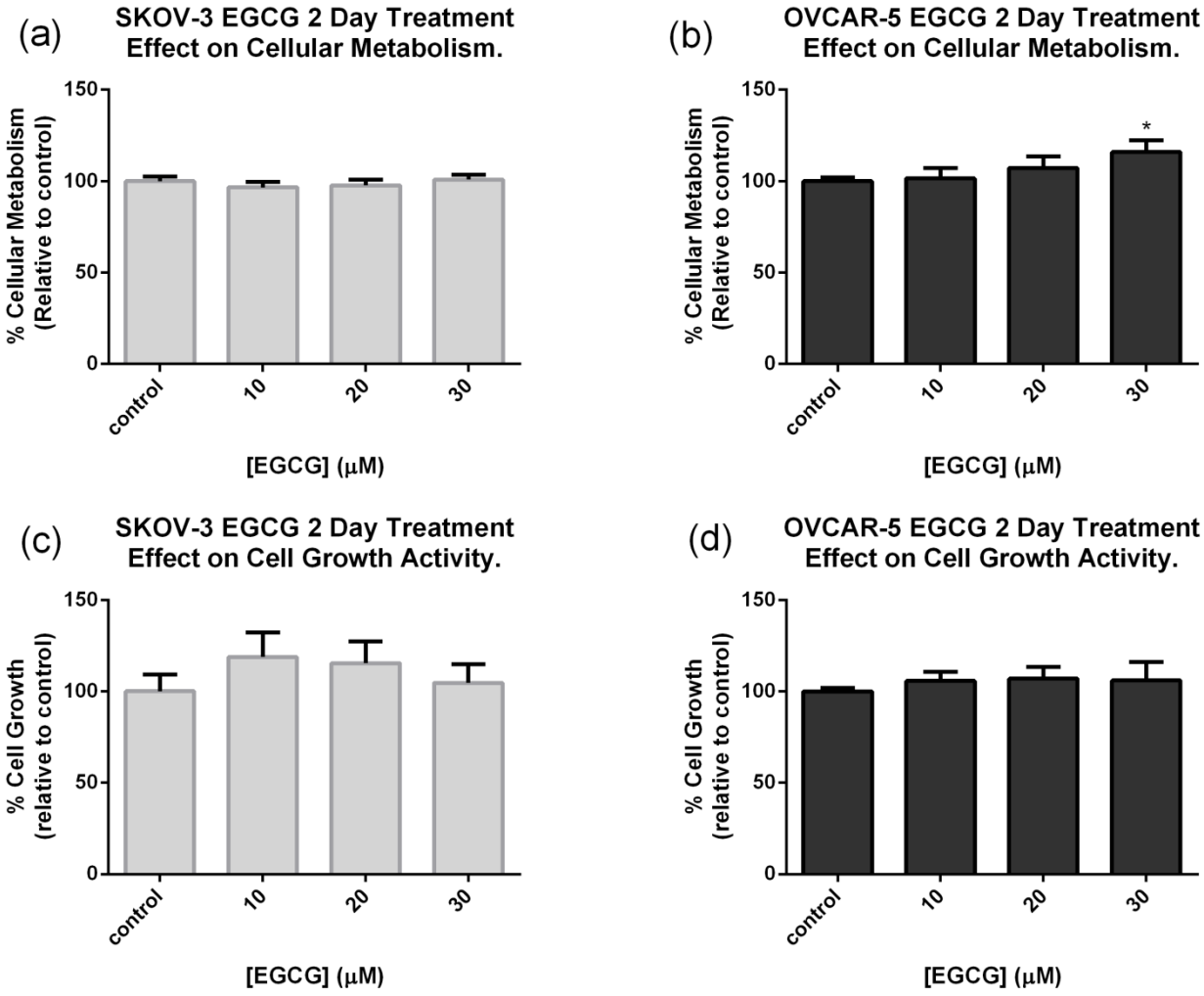

Figure 3.3. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of EGCG for 2 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

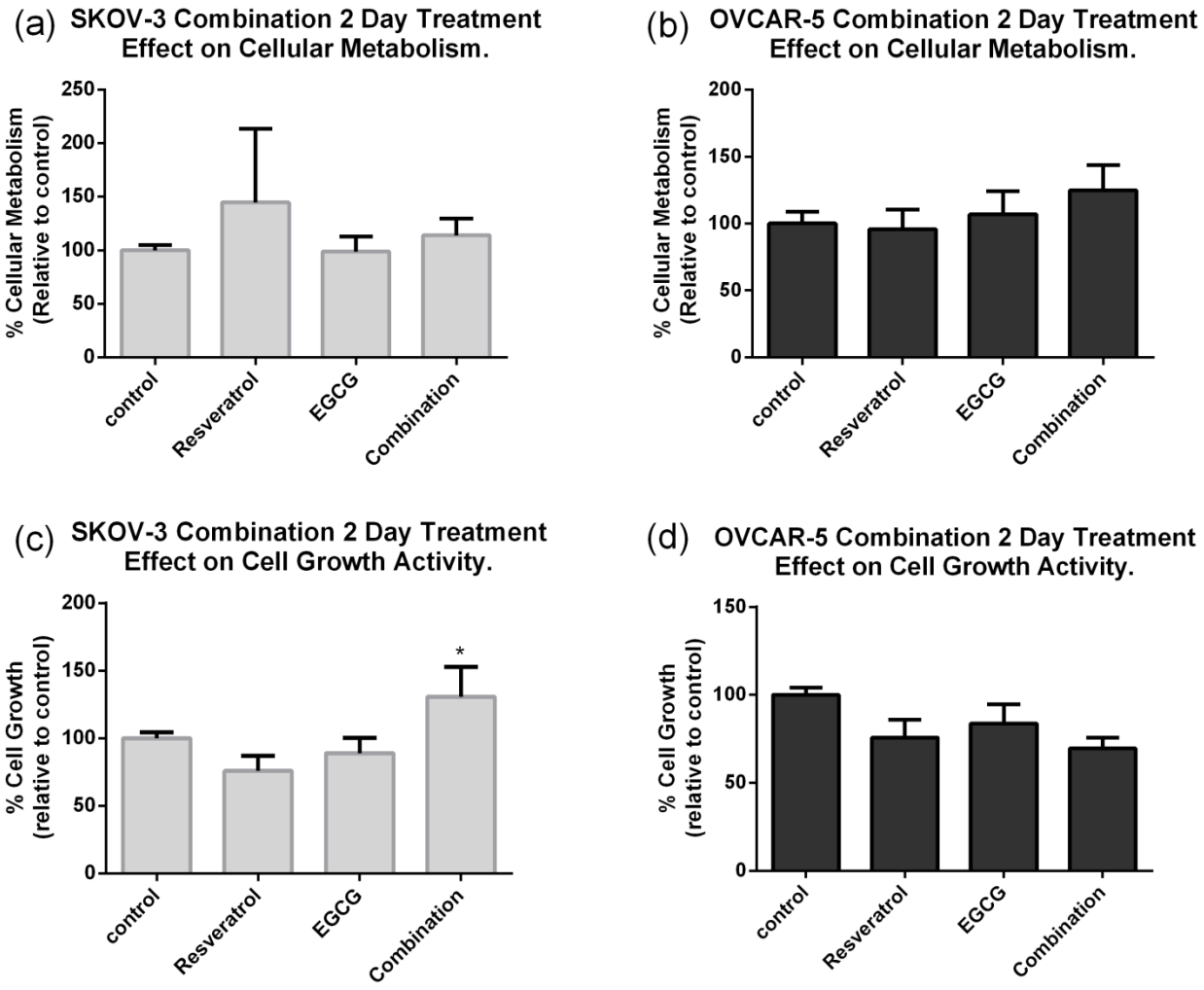

Figure 3.4. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with 20µM resveratrol, 20µM EGCG or 10µM resveratrol + 10µM EGCG (combination) for 2 days. Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

3.3.2 Four day treatment effects on Cellular Metabolism and Proliferation.

After four days of treatment with 10 and 30µM resveratrol cell metabolism of SKOV-3 was decreased by 22% (Fig. 3.5 a). About 14% growth reduction was also observed in SKOV-3 after four days of 30µM resveratrol treatment (Fig. 3.5 c).

Acetyl resveratrol did not affect cellular metabolism or cell growth in either cell line (Fig. 3.6 a, b, c, d) after four days of treatment. EGCG treatment at 10µM slightly increased metabolism of OVCAR-5 (Fig. 3.7) and 30µM EGCG slightly decreased SKOV-3 growth activity (Fig. 3.7 c).

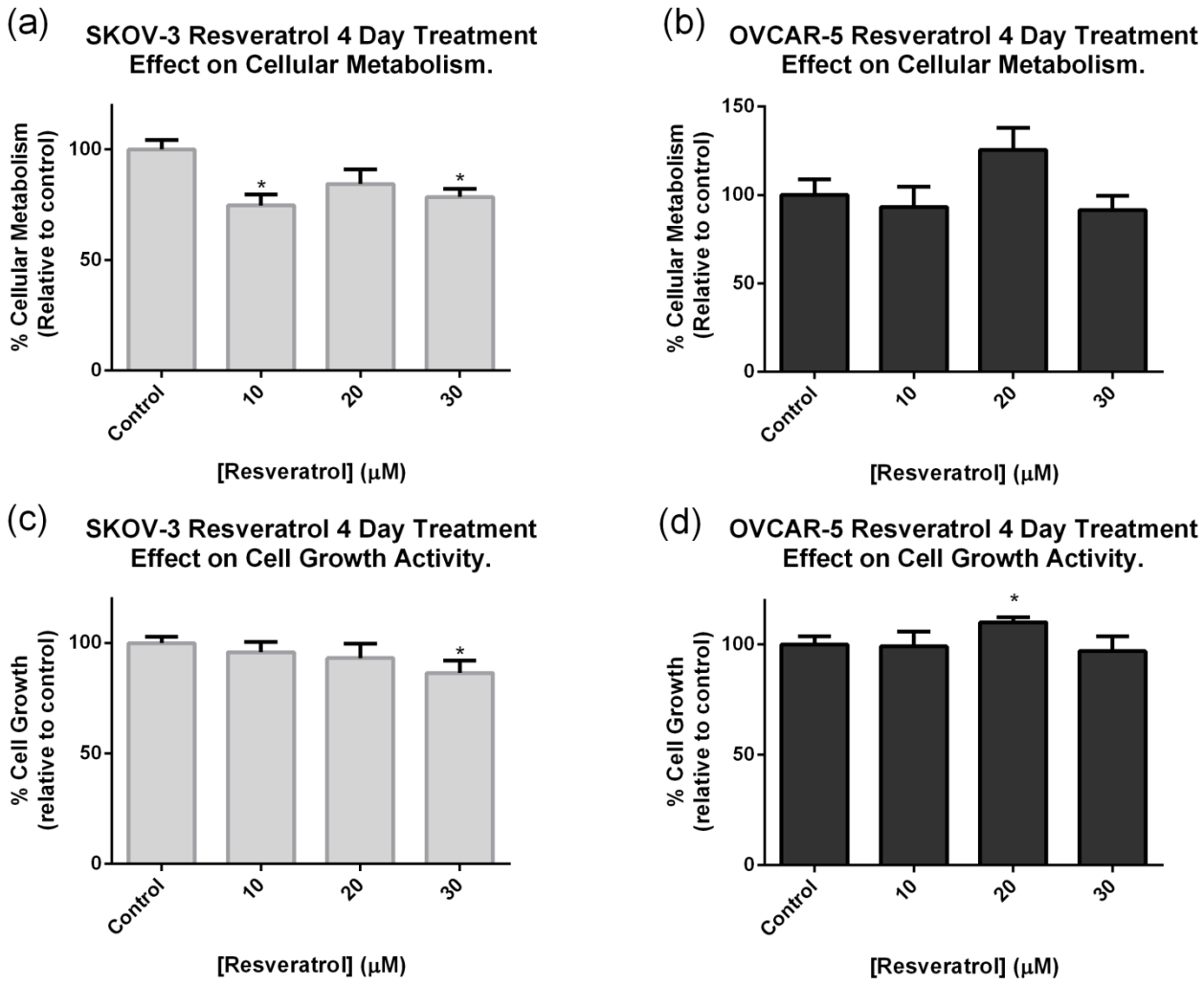

Figure 3.5. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of resveratrol for 4 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

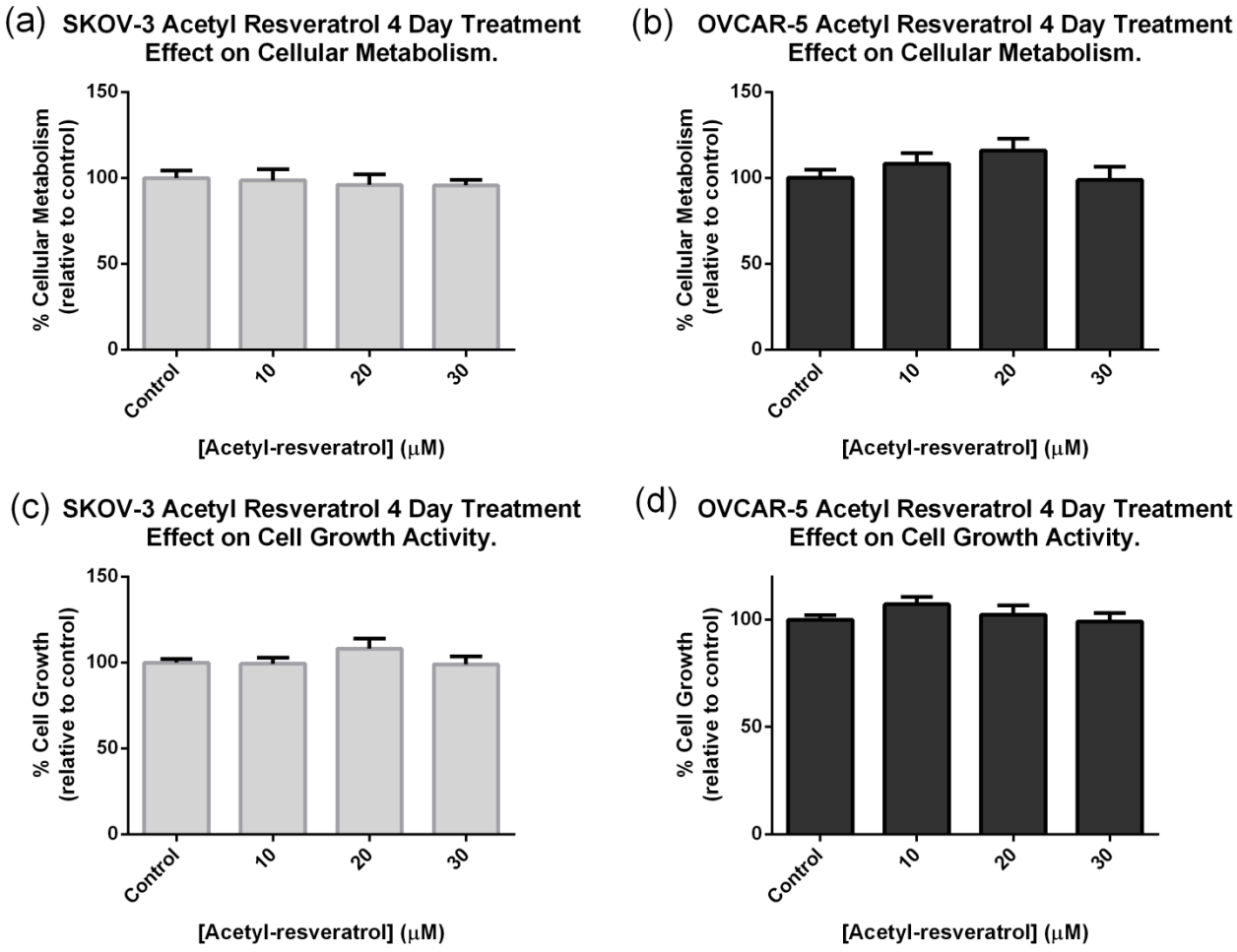

Figure 3.6. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of acetyl resveratrol for 4 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

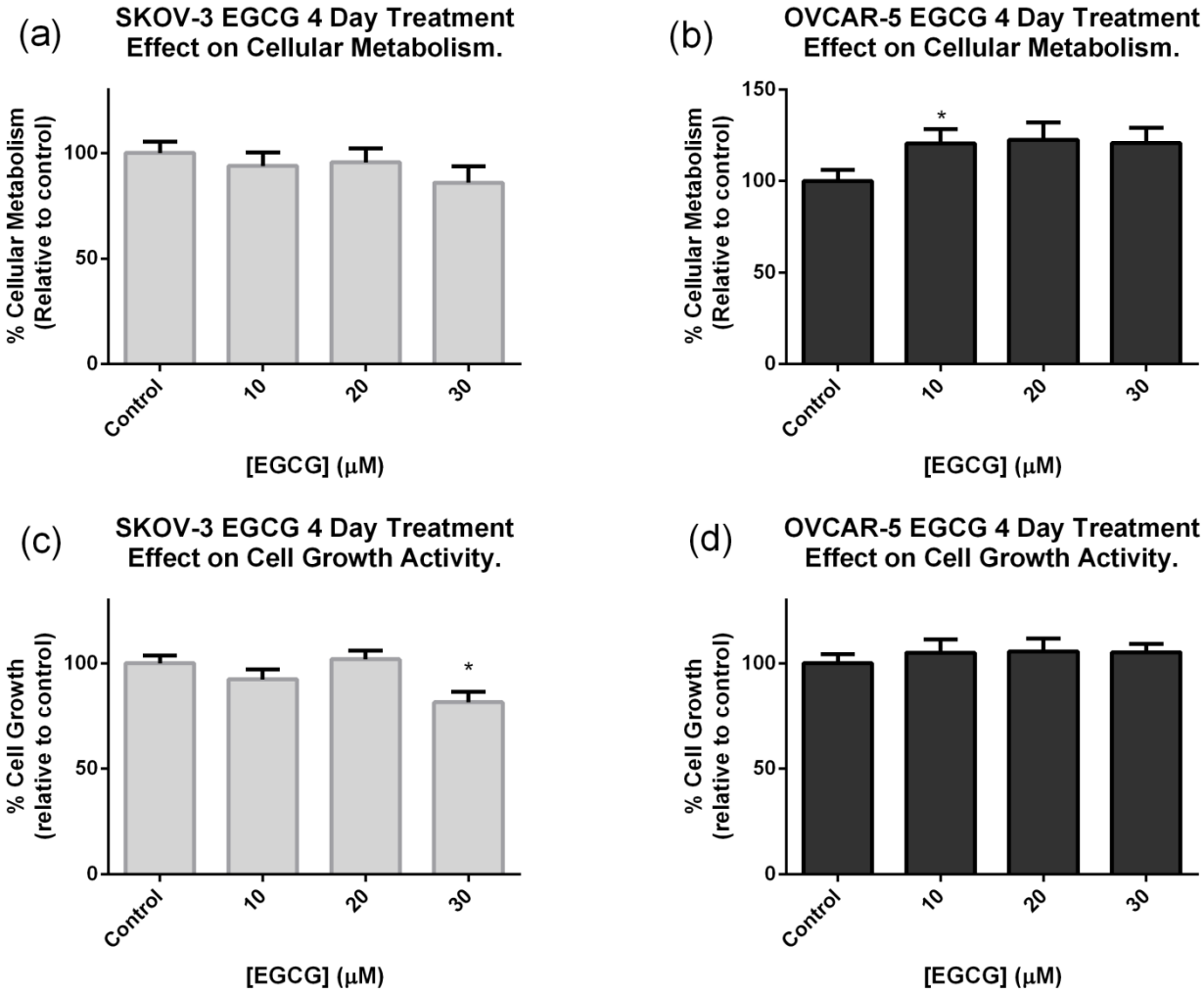

Figure 3.7. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of EGCG for 4 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

3.3.3 Six day treatment effects on Cellular Metabolism and Proliferation.

The SKOV-3 cell line was strongly affected after 6 days of treatment with resveratrol and acetyl resveratrol. Cellular metabolism was decreased by 36% and 39% after 20µM and 30µM resveratrol treatment respectively (Fig. 3.8 a). Cell growth reduced across all three doses of resveratrol (Fig. 3.8 c). Cell growth also decreased with all three treatment strengths of acetyl resveratrol (Fig. 3.9 c). In the combination experiments there is no significant difference between the single treatments of resveratrol or EGCG and the treatment with a combination of the two (Fig. 3.11 a, c).

The cellular metabolism of OVCAR-5 and cell growth were unaffected by 6 day treatment with resveratrol, acetyl resveratrol and the combination of 10µM EGCG with 10µM resveratrol (Fig. 3.8 b, d Fig. 3.9 b, d Fig. 3.11 b, d). Six days of treatment with EGCG did not affect either cell lines metabolic or growth activity (Fig. 3.10 a, b, c, d).

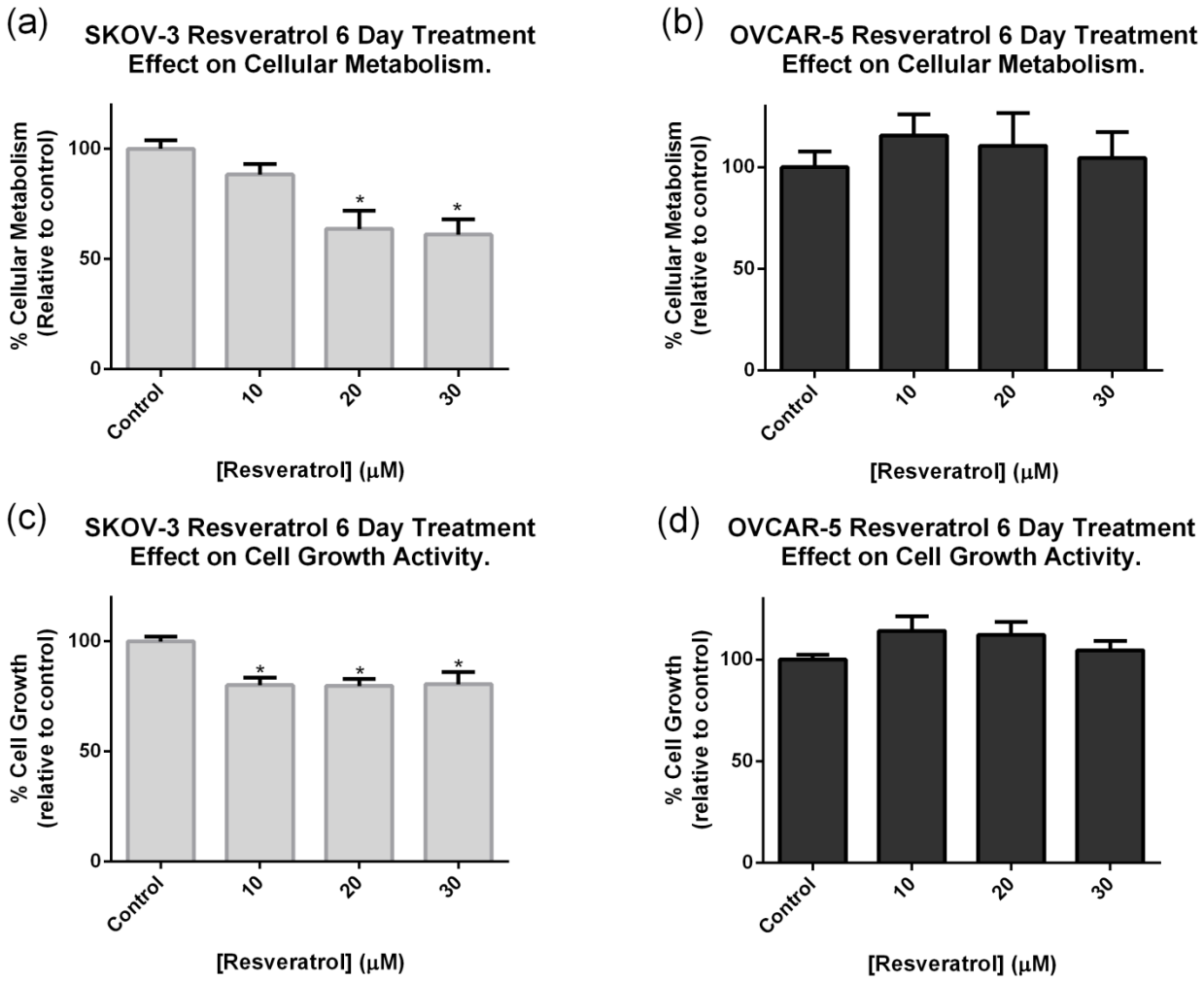

Figure 3.8. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of resveratrol for 6 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

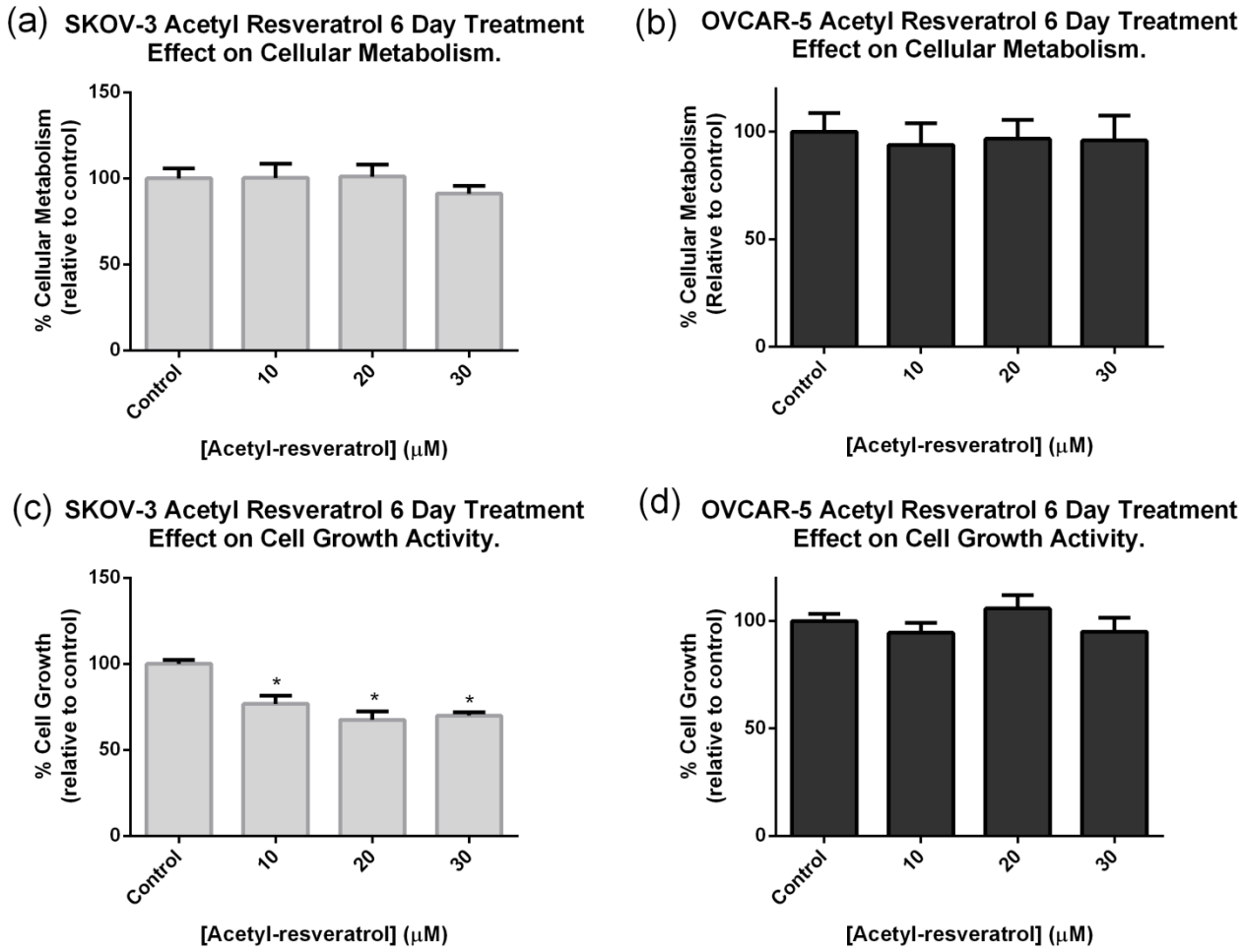

Figure 3.9. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of acetyl resveratrol for 6 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

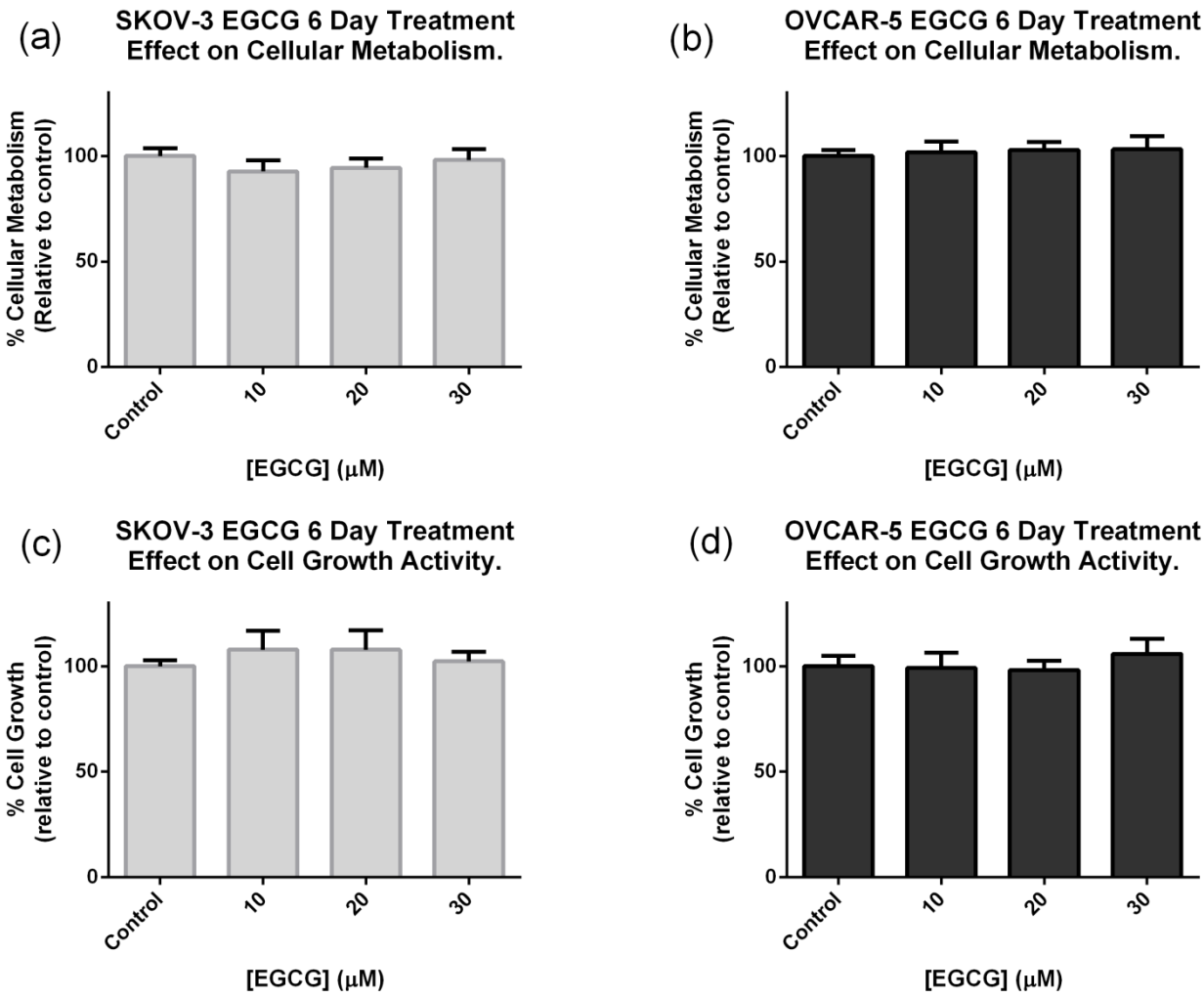

Figure 3.10. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of EGCG for 6 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

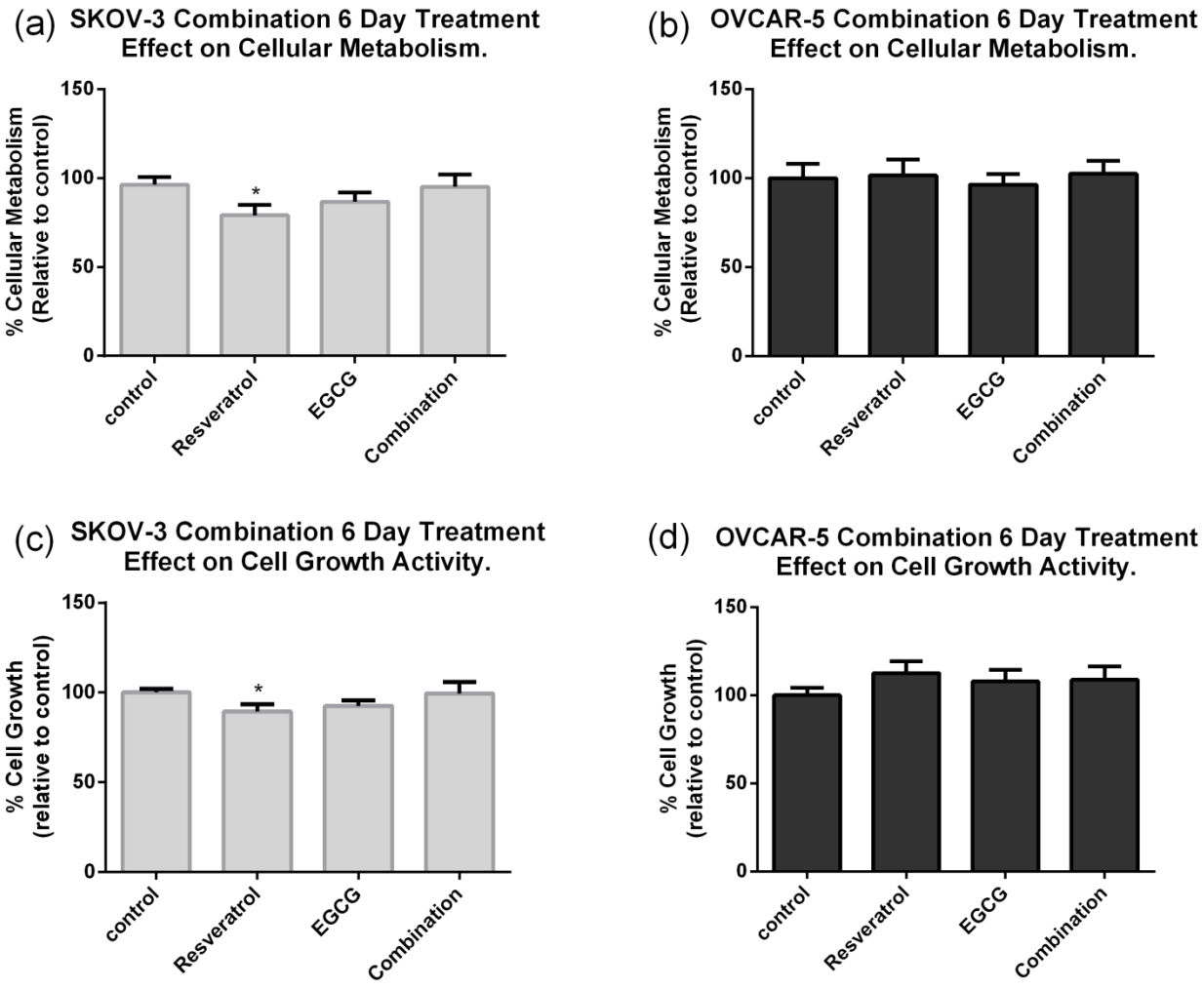

Figure 3.11. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with 20µM resveratrol, 20µM EGCG or 10µM resveratrol + 10µM EGCG (combination) for 6 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

3.3.4 Six Day Resveratrol Treatment effects on Spheroid/cluster morphology.

There were obvious morphological differences between the 3D cultures of the two cell lines. The SKOV-3 spheroids/clusters were over 10 fold larger than the OVCAR-5 ones (Fig. 3.12, Fig. 3.13). The OVCAR-5 spheroids/clusters were also quite uniform in size whereas the SKOV-3 spheroids/clusters were very irregular in shape. Resveratrol was the only treatment that had any obvious effect on the morphology of either cell line (other treatment effects not shown). The size and density of SKOV-3 spheroids/clusters noticeably decreased after treatment with 20 and 30 µM of resveratrol for 6 days compared to the control (Fig. 3.12 a, c, d). Although the OVCAR-5 spheroids/clusters were more dispersed after the 20 and 30 µM resveratrol treatments compared to the control (Fig. 3.13 a, c, d) there was no noticeable effect on the size or density of the spheroids/clusters.

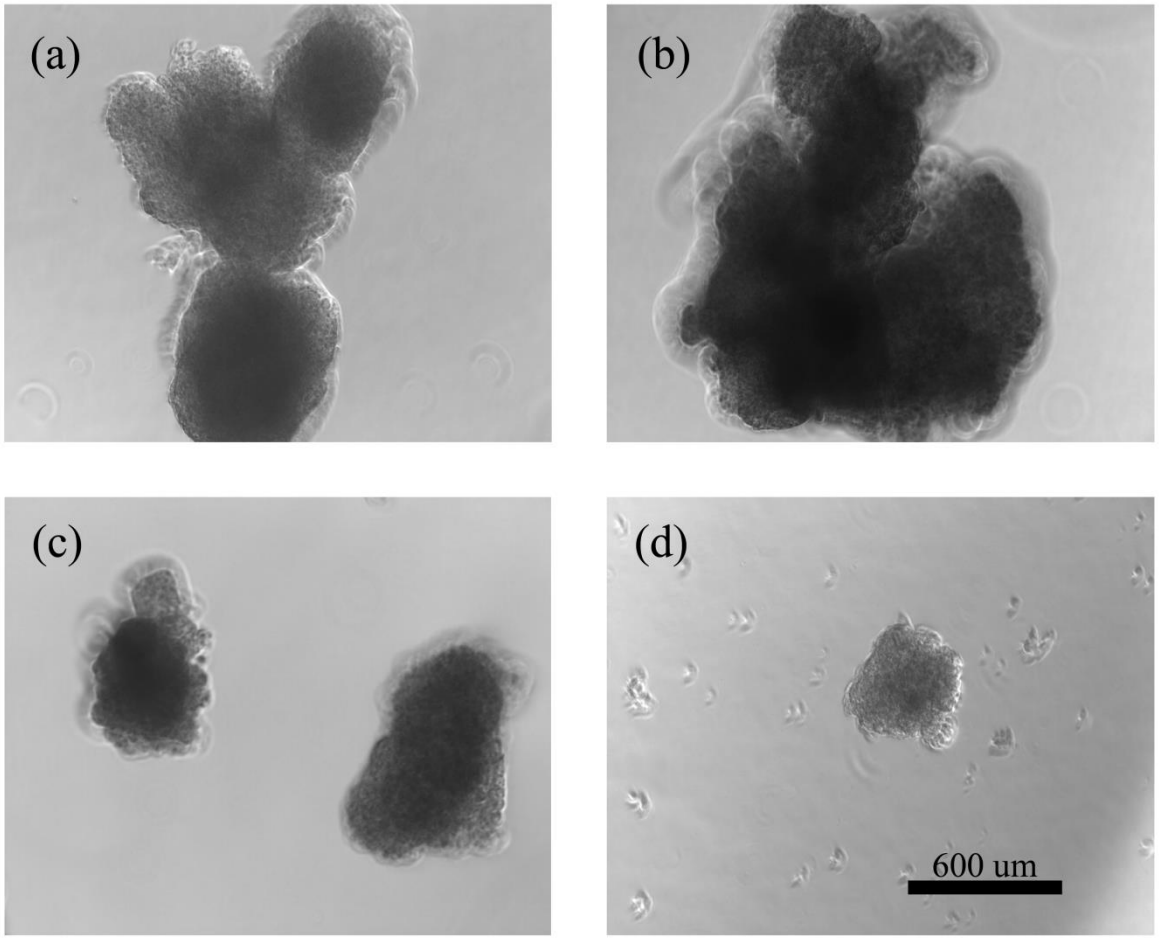

Figure 3.12. Morphology of SKOV-3 spheroids/clusters after 6 days treatment with resveratrol. (a) Control (b) 10µM resveratrol (c) 20µM resveratrol and (d) 30µM resveratrol. The morphology of the spheroids/clusters observably changed after treatment with 20 and 30µM resveratrol.

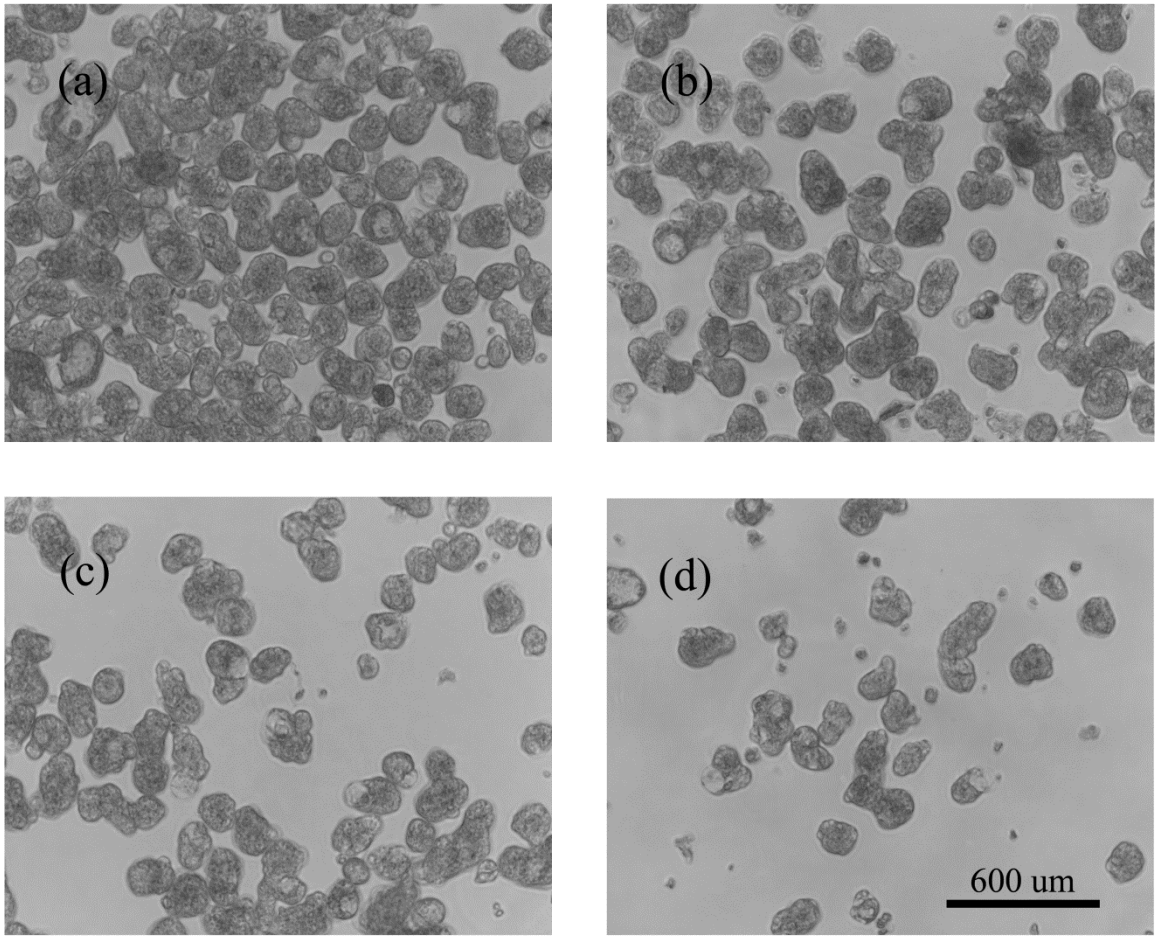

Figure 3.13. Morphology of OVCAR-5 spheroids/clusters after 6 days treatment with resveratrol. (a) Control (b) 10µM resveratrol (c) 20µM resveratrol and (d) 30µM resveratrol. The morphology of the spheroids/clusters did not observably change.

3.4 Discussion.

Many of the ovarian cancer chemotherapeutical agents currently in use are also known to be toxic to normal cells. To address this, it is highly important to utilise drugs that specifically induce cancer cell death whilst leaving healthy cells untouched. To this end, there are many natural food derivatives under investigation for their purported anti-cancer properties.

3.4.1 Resveratrol.

One such natural compound being investigated for potential anti-carcinogenic properties is the stilbenoid resveratrol. The most readily available food source with the highest concentration of resveratrol (2-40µM) is red wine, as it is present in the skin of red grapes (Kraft et al., 2009). Resveratrol has proven to be effective in decreasing cell proliferation in other epithelial-derived cancers such as skin, breast, prostate and lung (Stakleff et al., 2012). However, the results of those studies face difficulties when attempting to extrapolate to human *in vivo* conditions as they utilised either monolayer cell cultures or animal models.

Ovarian cancer's response to resveratrol is evidentially cell line dependant as OVCAR-5 is unaffected by the treatment whereas SKOV-3 displays a reduction in cellular metabolism and growth. This cell line dependency has also been observed in monolayer cultures of ovarian cancer (Cao et al., 2004, Lee et al., 2009) but to the best of my knowledge has not been demonstrated in a 3D culture.

The response of SKOV-3 is also time and dose dependant. The concentrations utilised in this study were chosen to fall within the amount of resveratrol found in red wine and what potentially may be present *in vivo* after imbibing a glass or two. After just 2 days treatment cell growth somewhat declined with the stronger treatments. It might seem unusual that cell proliferation decreased whilst metabolism did not. It is suggested that this is due to the size of the SKOV-3 spheroids/clusters. These spheroids/clusters are quite large therefore the surface area exposed to the alamar blue dye would not significantly change even though the cell number has decreased. Thus, the metabolic rate would appear unchanged

despite the loss of cells. After four days treatment with 30µM resveratrol metabolism and growth are reduced, then after six days metabolic rates have further declined with the stronger treatments (20 and 30µM) and cell growth is reduced with all three.

This is once again a novel outcome in the 3D structure and is also in accordance with previous monolayer studies, where SKOV-3's cell growth declined with stronger concentrations after one or two days and with lower concentrations after a longer time period (Raj et al. 2008; Lee et al., 2009). At four days of treatment with 10µM there is a reduction of metabolism but not proliferation, this is most likely an anomaly as it is not apparent at the higher concentration (20µM) with the same treatment time. Again one experiment had much lower results than the others, the P value is high and given a further replicate it is possible that the decrease would become non-significant.

The morphology of the spheroids/clusters after six days treatment with 20 and 30µM resveratrol is also affected with their size being dramatically reduced and their density also being observably reduced. These observations support the cell metabolism and growth results.

3.4.2 Acetyl resveratrol.

There is evidence however, that resveratrol has low bioavailability and the concentrations that reach the peritoneal cavity may be in the nanomolar range (Walle, 2011). One approach to address this is to utilise resveratrol analogues (Colin et al., 2009). Acetyl resveratrol is one such naturally occurring analogue where the hydroxyl groups of resveratrol are acetylated, this acetylation is purported to improve metabolic stability in humans as it does so in rats (Liang et al., 2013). Acetyl resveratrol is shown here to again be a cell line specific drug with OVCAR-5 being resistant and also time and dose dependant as there was no change in either metabolism or growth of SKOV-3 until the sixth day when only proliferation declined with all three drug concentrations. As above the reduction in growth and not cellular metabolism is attributed to the relatively large size of the spheroids/clusters formed.

3.4.3 EGCG.

EGCG a green tea constituent is another food derivative that is under investigation. In this study, overall EGCG demonstrated a lack of either time or dose dependent inhibition of the growth or metabolism of spheroids/clusters of either ovarian cancer cell line. In fact, OVCAR-5 appears to have a slightly positive response to the treatments; however this is not apparent as a dose or time dependant effect. SKOV-3 displayed a reduction in growth after four days treatment but the fact that the six day treatment did not display the same effect suggests that this may be an anomaly attributed to one experiment with particularly low readings and the P value is close to non-significant. Given a further replicate experiment, it is possible that the decrease would become non-significant. Although there is a plethora of studies revealing that EGCG is effective in reducing cell proliferation and or tumour size in cancers such as cervical, oesophagus, skin, and lung (Lu et al., 2002, Ahn et al., 2003, Lu et al., 2006) many of these use animal models and the differences between animals and humans in regards to metabolism make it difficult to extrapolate the results (Zhang et al., 2012). Huh et al., 2004 found that cell proliferation of ovarian cancer is supressed by EGCG; however the study was performed on 2-dimensional monolayers of cells *in vitro,* it is probable that EGCG is ineffectual in this study due to inadequate penetration of the clusters. The concentrations examined in this study are also much lower than many previous studies including Huh et al., 2004, who used 25-100µM EGCG to treat SKOV-3. The typical cup of green tea only contains 50-75mg of EGCG (Zhang et al., 2006) and of this circa 0.6µM is detected in human blood after consumption (Ahn et al., 2003), thus concentrations were chosen to better reflect amounts that could be expected to be present *in vivo*.

3.4.4 Resveratrol and EGCG combination.

It was hypothesised that resveratrol and EGCG can work synergistically in the 3D model. As resveratrol was able to downregulate cellular metabolism and proliferation in a 3D structure whereas EGCG was only effective on a 2D structure then resveratrol could sensitize the cluster allowing EGCG penetration. Thus combination experiments were undertaken, however no synergistic effect was observed.

Chapter 4.

Effects of Dietary Polyphenols on the Secretion of Angiogenic Proteins.

4.1 Introduction.

Data presented earlier in the thesis has shown that there is a reduction in cellular metabolism and growth in response to resveratrol and acetyl resveratrol at concentrations of 20 and 30 µM after four and six days of treatment. This chapter investigates the secretion of the angiogenic proteins Vascular Endothelial Growth Factor (VEGF) and Interleukin 8 (IL-8) as two possible factors involved in the effects of resveratrol and acetyl resveratrol.

4.1.1 Vascular Endothelial Growth Factor (VEGF).

4.1.1.1 Background.

VEGF is a homodimeric glycoprotein with a molecular weight of 45kDa; it plays a role in a diverse range of angiogenic activities. Angiogenesis is the physiological development of new blood vessels from pre-existing arteries, veins and lymphatic vessels. VEGF is a key regulator of angiogenesis during skeletal growth, embryogenesis and cyclical ovarian function (Ferrara et al., 2003). VEGF stimulates the proliferation and survival of vascular endothelial cells (Ferrara et al., 2003).

4.1.1.2 VEGF and Cancer.

For the development and growth of solid tumours, angiogenesis is of fundamental importance and as stated previously VEGF is essential to this process (Shimizu et al., 2010). Angiogenesis is significant in tumour progression as it supplies oxygen, nutrients, growth factors and hormones, basically, everything that a tumour needs to flourish (Hicklin and Ellis, 2005). VEGF has also been shown to mediate vessel permeability, this is also crucial in cancer development as this enables the metastasis of the malignant cells (Hicklin and Ellis, 2005). The ‘switch’ to an angiogenic phenotype is thought to be a trademark of the

malignancy process and the overexpression of VEGF is related to advanced stages of the disease and a poor prognosis for patients in an assortment of cancers (Hicklin and Ellis, 2005). There is also evidence that tumour cells express VEGF receptors, this expression has been suggested to produce an autocrine loop, which enables these cells to avert apoptosis when VEGF is present (Hicklin and Ellis, 2005).

4.1.1.3 VEGF and Ovarian Cancer.

It has been suggested that the ability of VEGF to induce vascular permeability is vital for the formation of malignant ascites in ovarian cancer (Mesiano et al., 1998). Overexpression of VEGF has been observed in ovarian cancer cell lines and has been linked to cell proliferation (Cao et al., 2004, Park et al., 2007). The expression level of VEGF in the ascites and primary tumours directly correlates with a poor prognosis (Yu et al., 2013). Together, these findings suggest a strong possibility that VEGF is important in tumour growth in ovarian cancer.

4.1.2 Interleukin-8 (IL-8).

4.1.2.1 Background.

The pro-inflammatory chemokine IL-8 is a 10 kDa glycoprotein. It is induced by inflammatory stimuli and promotes a series of physiological responses that are needed for cell migration and phagocytosis (Hebert and Baker, 1993). IL-8 is primarily responsible for the chemoattraction and activation of neutrophils at sites of inflammation (Holmes et al., 1991). It stimulates neutrophil degranulation and inhibits their adherence to activated endothelial cells (Holmes et al., 1991).

4.1.2.2 IL-8 and Cancer.

IL-8 is purported to be essential for tumour inflammation, angiogenesis and growth in many cancers (Sparmann and Bar-Sagi, 2004). Its overexpression has been identified in a variety of cancers including breast, colon, cervical, gastric and lung cancer (Xie, 2001). The identification of receptors of the chemokine on tumour cells has led to the suggestion that IL-8 can also form an autocrine loop and thus, sustain endothelial and cancer cell survival (Xie, 2001). The inhibition of IL-8 secretion resulting in decreased cell proliferation has been established in melanoma, cervical, pancreatic and colon cancers (Xie, 2001,

Sparmann and Bar-Sagi, 2004,). IL-8 signalling has also been shown to be inducible by drug treatment and result in resistance of chemotherapies in some cancers (Waugh and Wilson, 2008).

4.1.2.3 IL-8 and Ovarian Cancer.

Overall, there have been relatively few investigations into the relationship between ovarian cancer and IL-8. However, there is evidence that in some ovarian cancer cell lines the expression level of IL-8 is correlated with the aggressiveness of the disease (Huang et al., 2000). Suppression of IL-8 secretion has also been linked to the attenuation of tumour growth and angiogenesis of ovarian cancer (Huang et al., 2000, Waugh and Wilson, 2008). These studies indicate that IL-8 may potentiate ovarian cancer cell growth and is a possible target of the natural drug treatments.

4.1.3 Aims of chapter 4.

This chapter aims to investigate whether VEGF and/or IL-8 secretion is attenuated by resveratrol, acetyl resveratrol or EGCG in the ovarian cancer cell lines SKOV-3 and OVCAR-5.

4.2 Methods and Materials.

Experimental 3D spheroid cell cultures -See Chapter 2 for full method and materials.

VEGF and IL-8 ELISA -See Chapter 2 for full method and materials.

BCA Protein Assay -See Chapter 2 for full method and materials.

4.3 Results.

4.3.1 VEGF

4.3.1.1 VEGF secretion in basal conditions.

The concentration of VEGF present in cell media after two, four or six days of drug treatment was assessed using the human VEGF ELISA Kit. An 8 point standard curve was created and the amount of VEGF in each sample was found by comparing absorbance of the samples to the standard curve. The concentrations were then normalised by dividing the amount by the total protein of the sample obtained using the BSA protein assay.

The secretion of VEGF was approximately four times higher from the SKOV-3 cell cultures than from OVCAR-5 cultures (Fig. 4.1, 4.2, 4.3, 4.4). The variation of base secretion for SKOV-3 was between 1.3 and 7.4 ng/ml/mg (Fig. 4.1 c Fig. 4.3 c). For OVCAR-5, the variation was from 0.4 to 1.1 ng/ml/mg (Fig. 4.1 b, f).

4.3.1.2 Resveratrol Treatment Effects on Secreted VEGF Concentrations.

The response to resveratrol treatment was cell line dependant with only SKOV-3 VEGF levels being affected significantly (Fig. 4.1). A time and dose dependency was also evident as after two days there was no significant effect, however after four and six days of treatment there was a trend of decreasing VEGF concentration with increasing dosage (Fig. 4.1 a, c, e) and the highest concentration of resveratrol resulted in a 41 and 54% decline in four and six day VEGF concentrations respectively when compared to the control (Fig. 4.1 c, e).

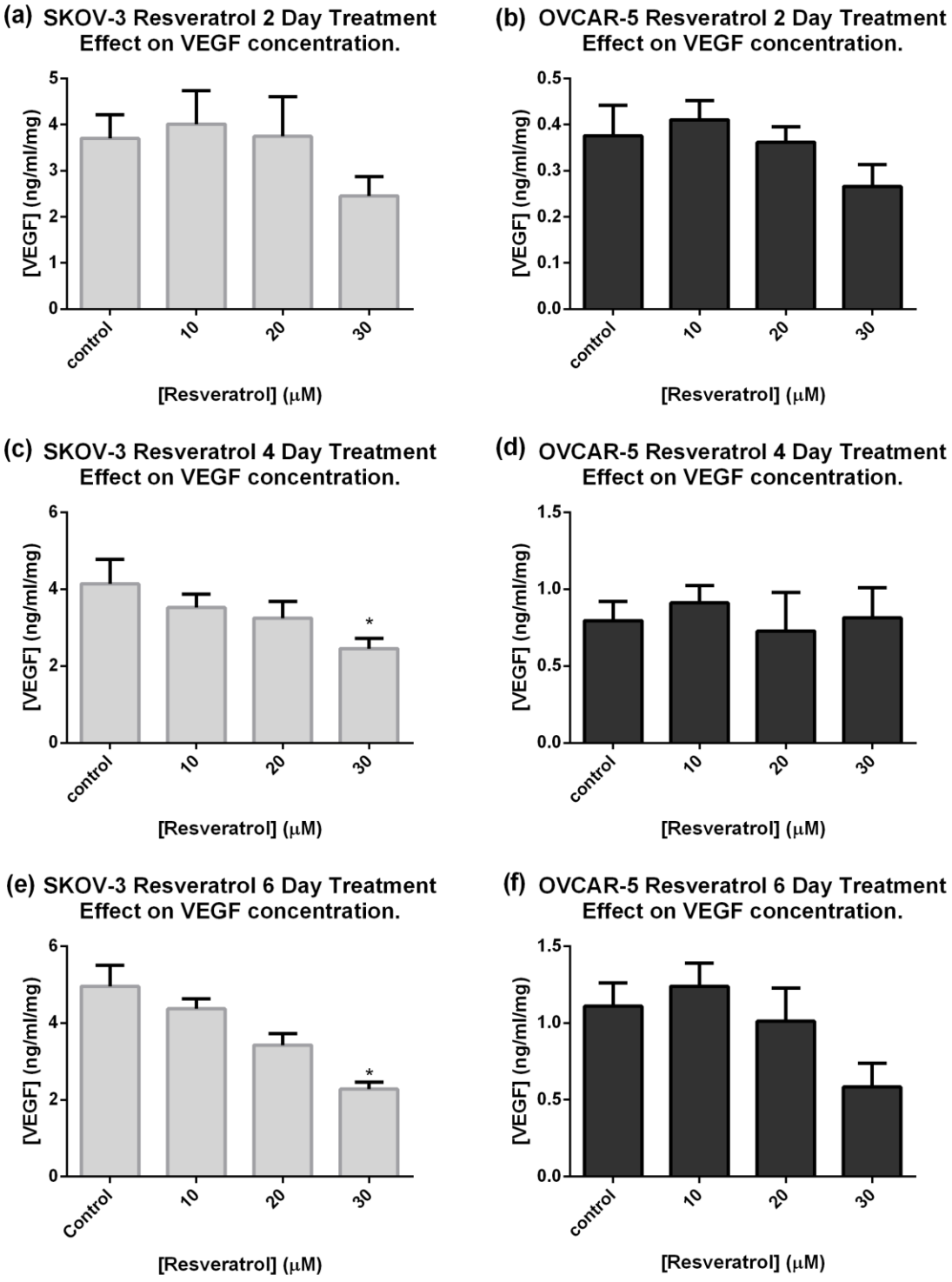

Figure 4.1. VEGF supernatant concentration (as determined by ELISA and expressed as ng/ml VEGF/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of resveratrol for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

4.2.1.3 Acetyl resveratrol Treatment Effects on Secreted VEGF Concentrations.

SKOV-3 was again the cell line that showed a response to the treatment (Fig. 4.2). The effect was dose dependant with the strongest dose tested 30µM decreasing VEGF secretion by 49% after four days treatment (Fig. 4.2 c) and by 58% after 6 days treatment (Fig. 4.2 e). There was also a time dependant effect as the decrease was at its greatest after six days treatment, whilst there was no significant suppression after two days (Fig.4.2 a, c, e).

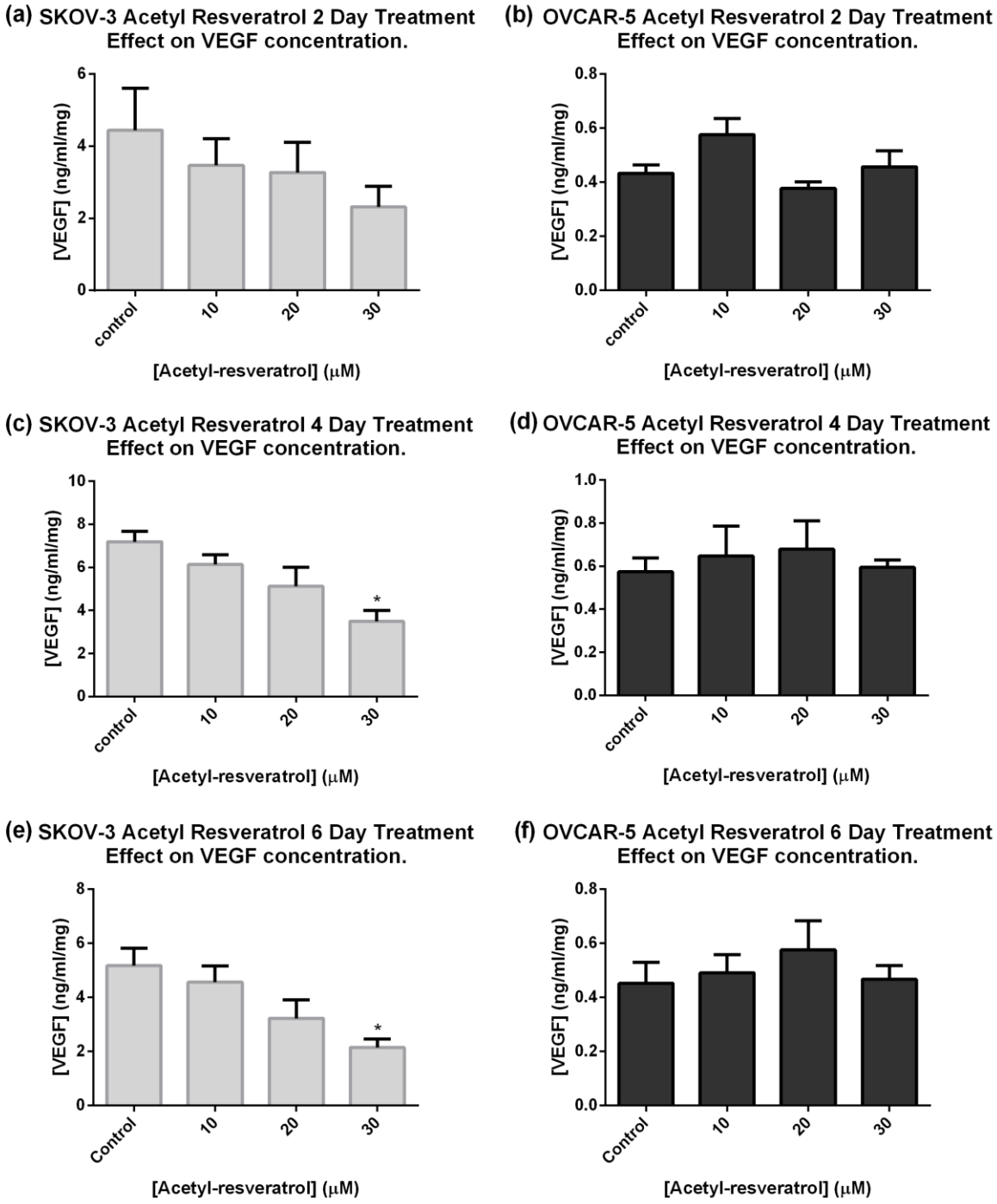

Figure 4.2. VEGF supernatant concentration (as determined by ELISA and expressed as ng/ml VEGF/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of acetyl resveratrol for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

4.2.1.4 EGCG Treatment Effects on Secreted VEGF Concentrations.

There was no significant effect on VEGF secretion in either cell line by EGCG (Fig. 4.3).

4.2.1.5 Combination Treatment Effects on Secreted VEGF Concentrations.

Combining resveratrol and EGCG had no effect on VEGF concentrations after two or six day treatments (Fig. 4.4).

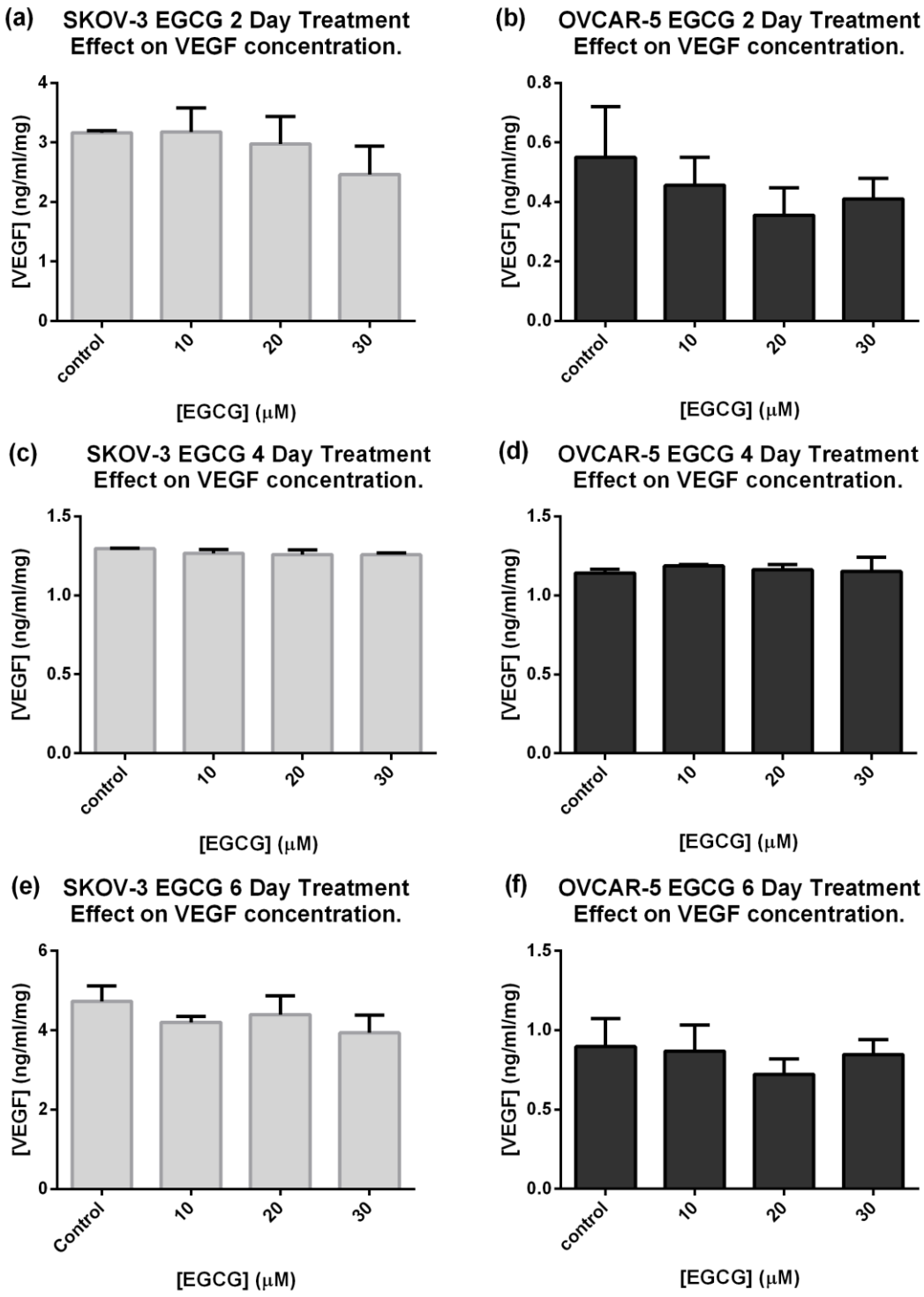

Figure 4.3. VEGF supernatant concentration (as determined by ELISA and expressed as ng/ml VEGF/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of EGCG for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

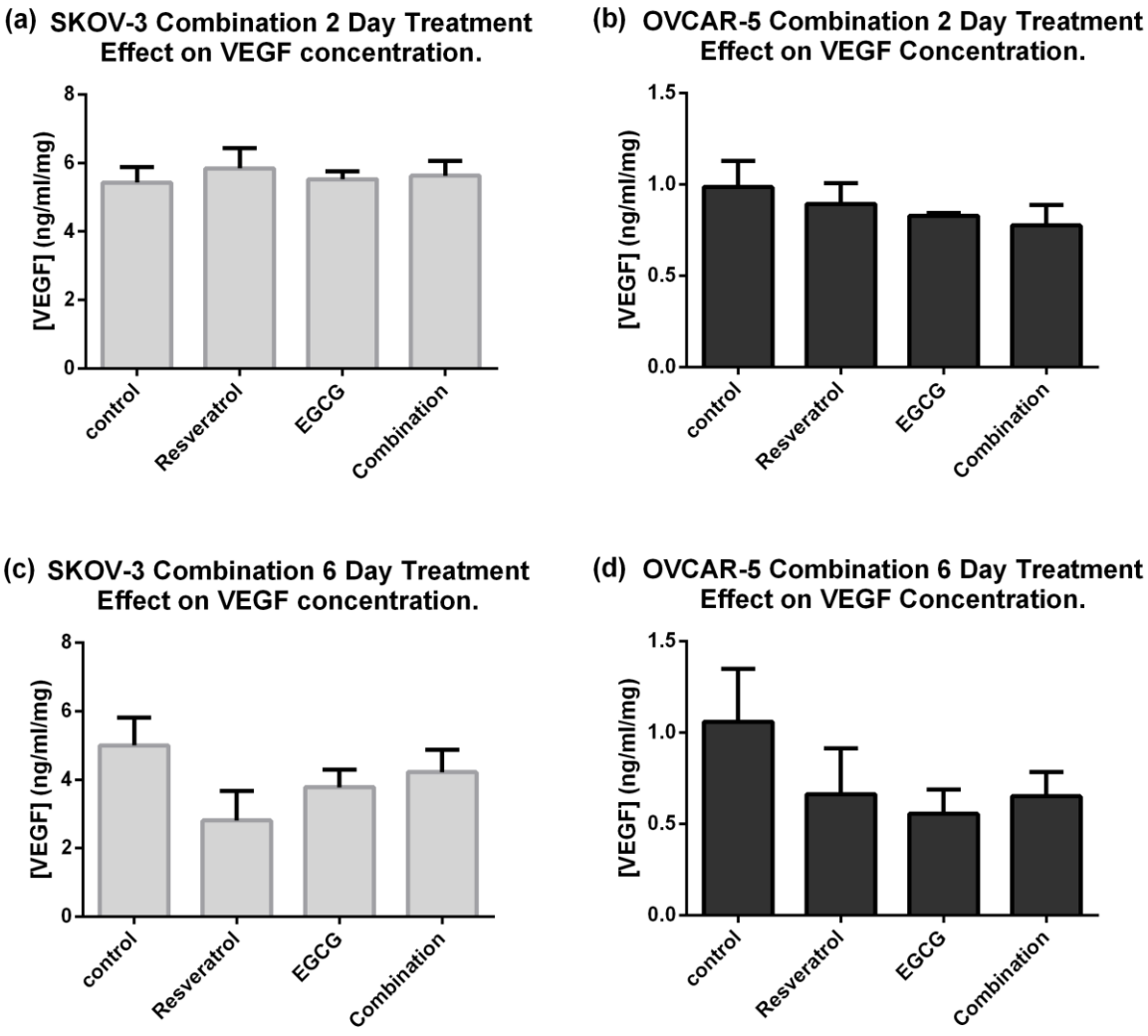

Figure 4.4. VEGF supernatant concentration (as determined by ELISA and expressed as ng/ml VEGF/mg total cell protein content) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with 20µM resveratrol, 20µM EGCG or 10µM resveratrol + 10µM EGCG (combination) for 2 days (a, b) or 6 days (c, d). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

4.3.2 IL-8

4.3.2.1 IL-8 secretion in basal conditions.

The concentration of IL-8 present in cell media after two, four or six days of drug treatment was assessed using a human IL-8 ELISA Kit. An 8 point standard curve was created and the amount of IL-8 in each sample was found by comparing the absorbance of the samples to the standard curve. The concentrations were then normalised by dividing the amount by the total protein of the sample obtained using the BSA protein assay.

The basal secretion of IL-8 from SKOV-3 cells (typically between 1 and 2 ng/ml/mg) was much greater than that from OVCAR-5 which went from below detectable concentrations up to 0.1 ng/ml/mg (Fig. 4.5, 4.6, 4.7, 4.8).

4.3.2.2 Resveratrol Treatment effects on secreted IL-8 concentrations.

Unlike the VEGF secretion, IL-8 concentration increased with resveratrol treatment (Fig. 4.5). The increase in secreted IL-8 in SKOV-3 was time and dosage dependent. Two days of resveratrol treatment gave no significant effect, however after 4 and 6 days treatment with 10µM resveratrol IL-8 concentrations doubled and after 20µM or 30µM treatment IL-8 levels tripled (Fig. 4.5 a, c, d). OVCAR-5 IL-8 concentrations also increased from undetectable levels after 2 days treatment to between 0.1 and 0.2 ng/ml/mg after 6 days treatment (Fig. 4.5 b, f). There was only one statistically significant result in OVCAR-5 IL-8 levels where the level increased 400% after 4 days treatment with 10µM resveratrol, the other increases after stronger doses were not significant due to the large standard error of the mean (Fig. 4.5 d, f).

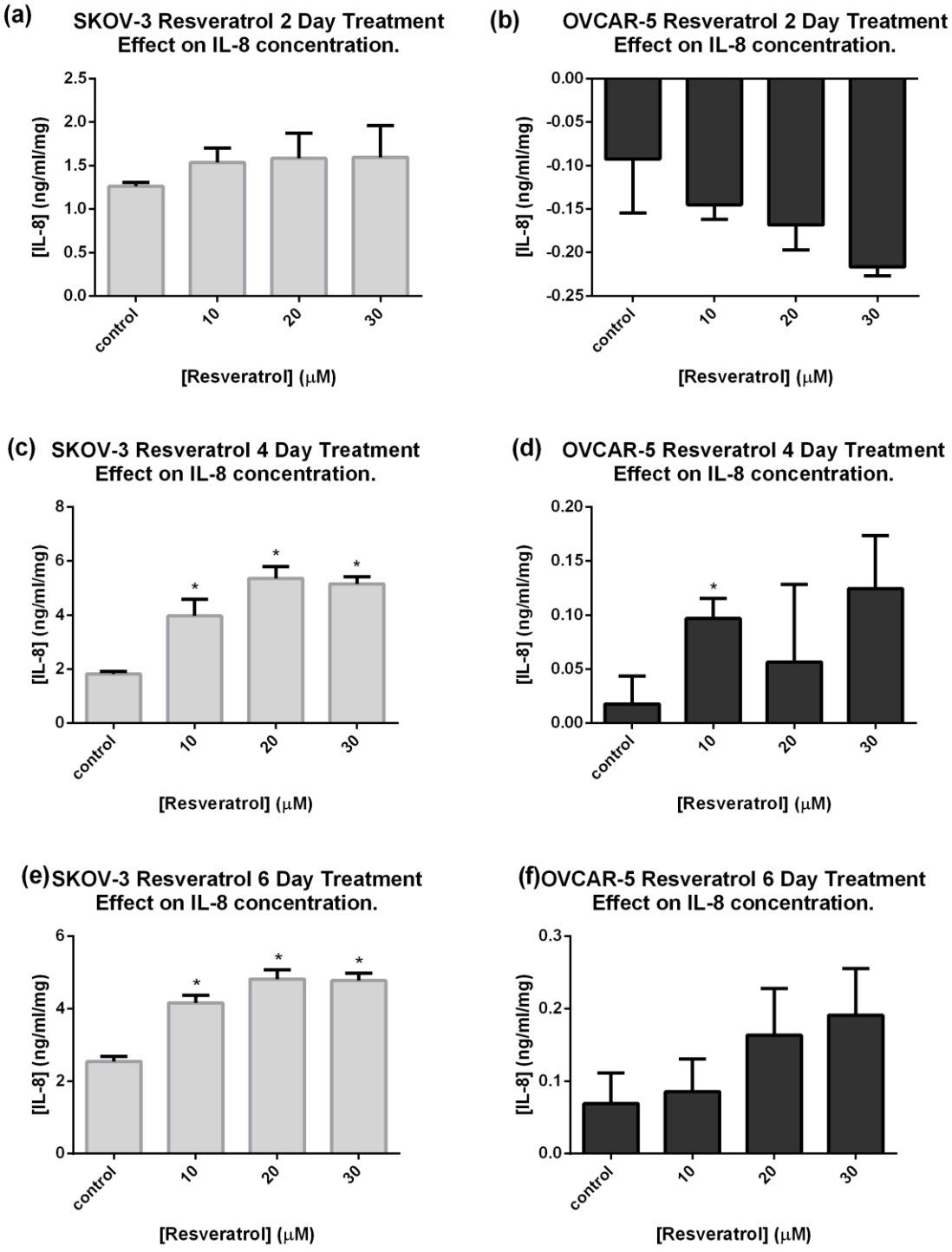

Figure 4.5. IL-8 supernatant concentration (as determined by ELISA and expressed as ng/ml IL-8/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of resveratrol for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

4.3.2.3 Acetyl Resveratrol Treatment effects on secreted IL-8 concentrations.

Both cell lines appear to increase IL-8 secretion in response to acetyl resveratrol treatment although OVCAR-5's response was not significant due to quite large standard errors (Fig. 4.6). Acetyl resveratrol's effect on SKOV-3 IL-8 concentrations was time dependent as only the 6 day treatment elicited a significant increase. IL-8 secretion was doubled across all doses (Fig. 4.6 e).

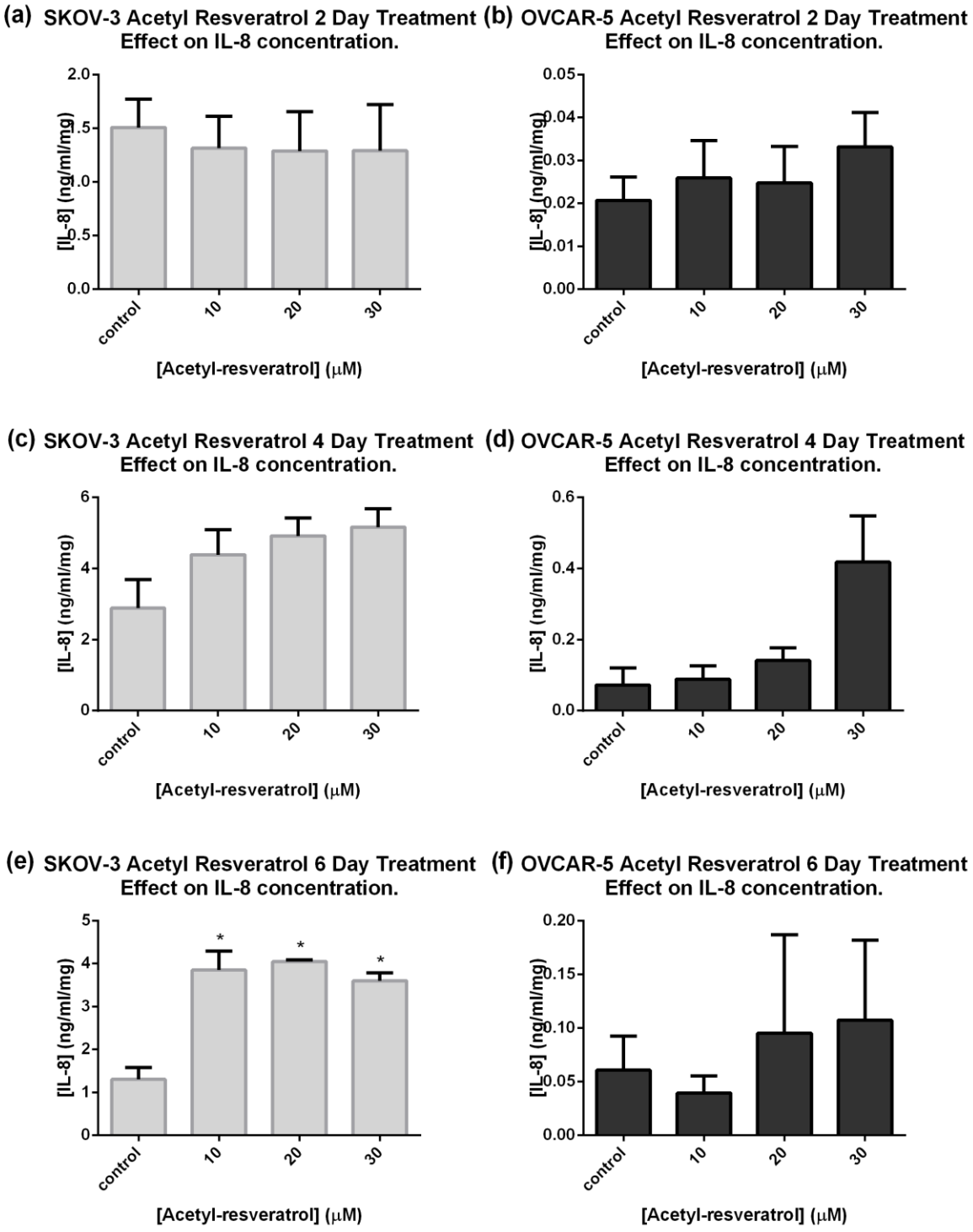

Figure 4.6. IL-8 supernatant concentration (as determined by ELISA and expressed as ng/ml IL-8/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of acetyl resveratrol for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

4.3.2.4 EGCG Treatment Effects on Secreted IL-8 Concentrations.

EGCG did not affect either cell line at any of the doses tested (Fig. 4.7).

4.3.2.5 Combination Treatment Effects on Secreted IL-8 Concentrations.

The combination of resveratrol and EGCG did not have any significant effect on either cell line (Fig. 4.8). However, EGCG alone increased IL-8 secretion after 2 days treatment (Fig. 4.8 a) although this result may be an anomaly as the effect was not seen in either the 6 day combination experiment or in the EGCG experiments (Fig. 4.7, Fig. 4.8).

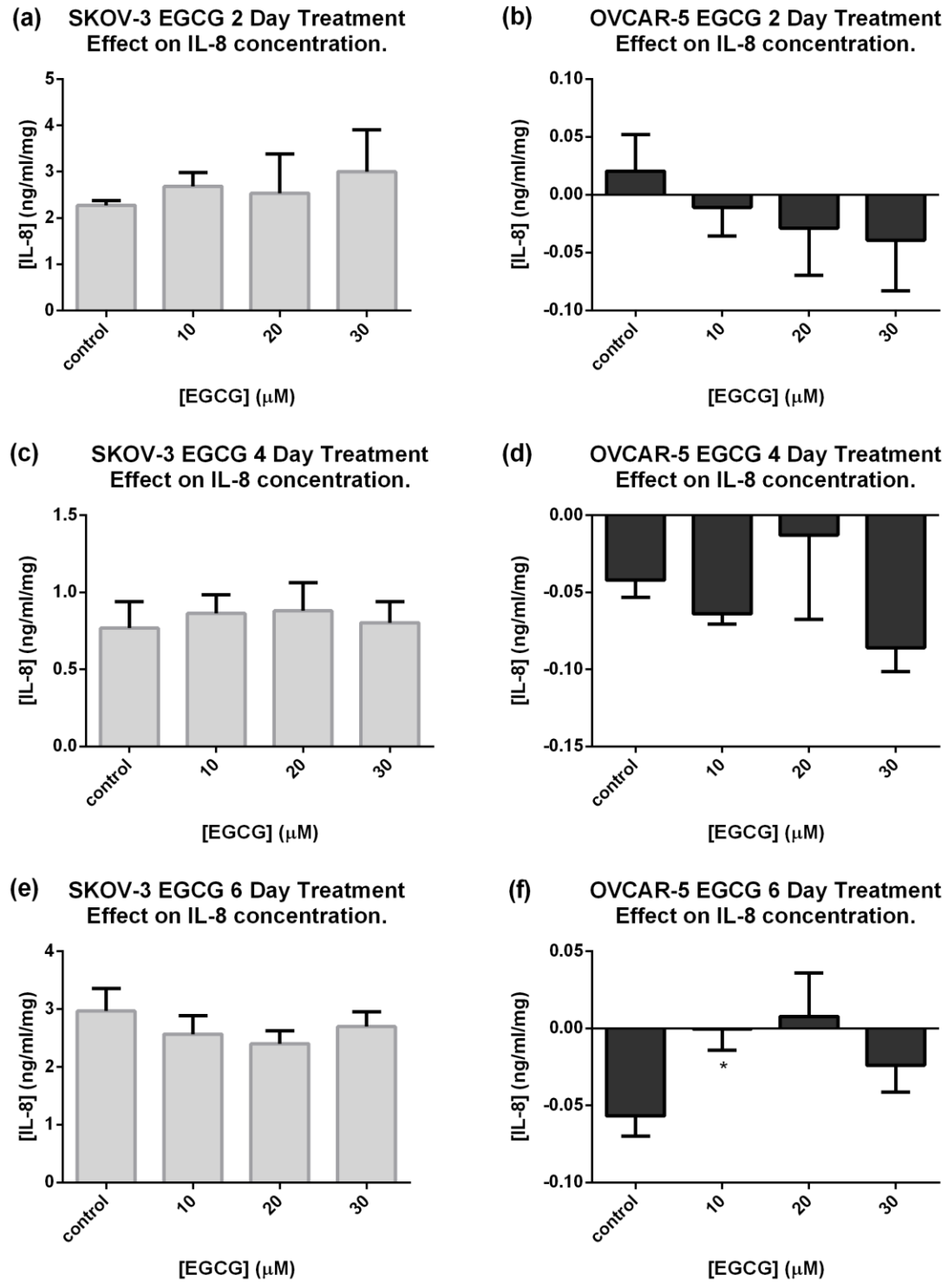

Figure 4.7. IL-8 supernatant concentration (as determined by ELISA and expressed as ng/ml IL-8/mg total cell protein content) of SKOV-3 (a, c, e) and OVCAR-5 (b, d, f) spheroids/clusters treated with various concentrations of EGCG for 2 days (a, b), 4 days (c, d) or 6 days (e, f). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test $P<0.05$) relative to the control.

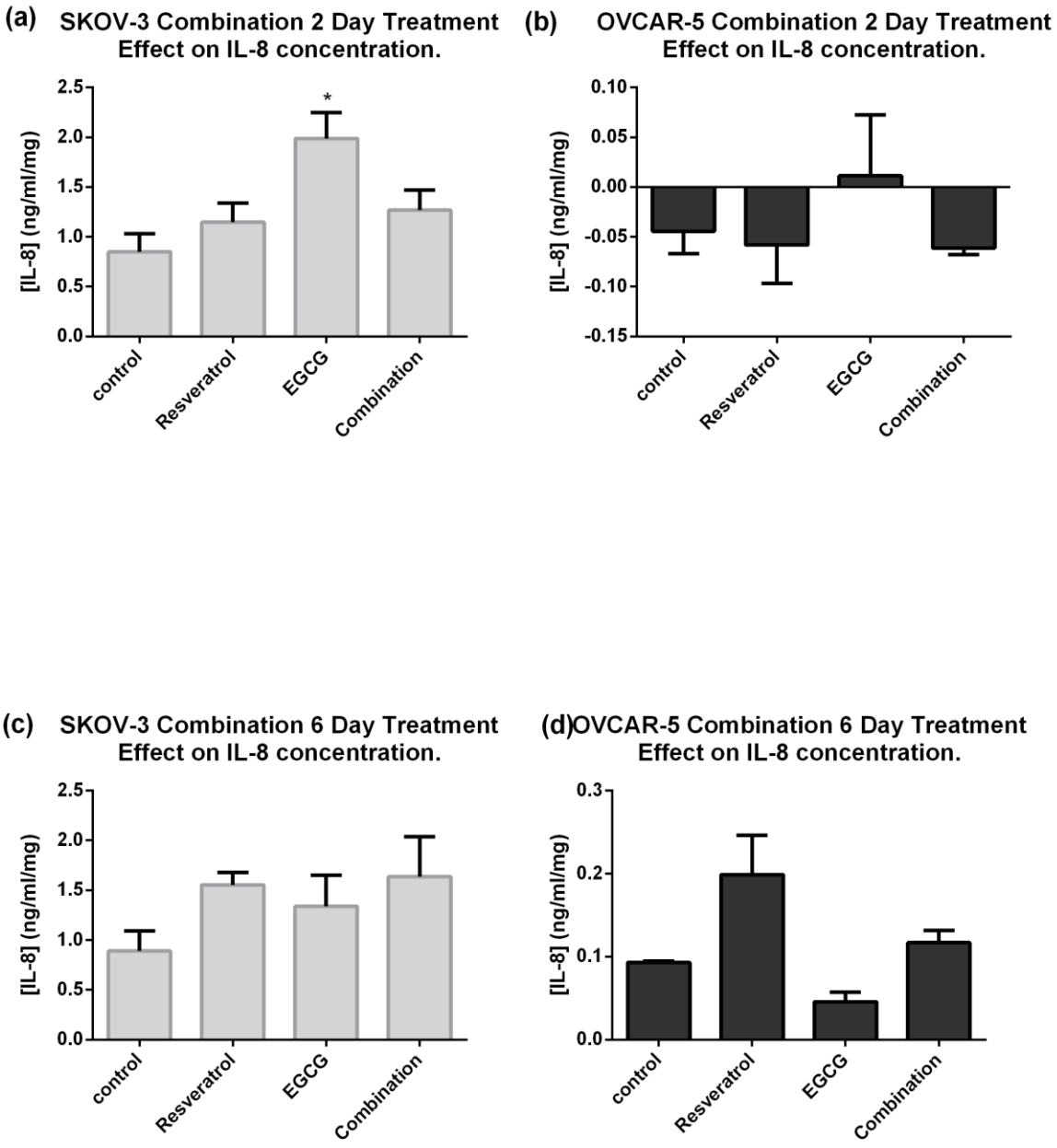

Figure 4.8. IL-8 supernatant concentration (as determined by ELISA and expressed as ng/ml IL-8/mg total cell protein content) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with 20µM resveratrol, 20µM EGCG or 10µM resveratrol + 10µM EGCG (combination) for 2 days (a, b) or 6 days (c, d). Data are from three independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

4.4 Discussion.

It has been established that VEGF production is elevated in tumour cells when compared to homeostatic cells and that it is pathologically vital for angiogenesis in many cancers (Shimizu et al., 2010). Increased VEGF production by ovarian cancer cells has also been associated with poorer prognoses in sufferers (Yu et al., 2013). The correlation between IL-8 secretion and prognosis of cancer patients is not so well defined. In breast cancer, IL-8 concentration is possibly a novel maker for tumour aggressiveness in estrogen-receptor (ER) positive cells; however it is ER-positive cancers that have the better prognosis (Freund et al., 2003). For cervical cancer, elevated IL-8 expression (greater than 1 ng/mg protein) is correlated with an extremely poor prognosis (Fujimoto et al., 2000). In ovarian cancer one study found that it was not a predictor of prognosis (Lane et al., 2011), whilst another linked high production of IL-8 to paclitaxel resistance and thus poor prognosis (Penson et al., 2000). The attenuation of the two proteins has also been linked to cell apoptosis and tumour growth reduction (Sparmann and Bar-Sagi, 2004, Shimizu et al., 2010, Mikula-Pietrasik et al., 2012).

The current study reveals that VEGF secretion is much higher than IL-8 secretion overall, this is in line with other studies with typical basal secretion of VEGF being 1-3 ng/ml (Park et al., 2007, Dann et al., 2009) and basal IL-8 production between 0 and 400 pg/ml (Freund et al., 2003, Trompezinski et al., 2003). In breast and other ovarian cancer lines it has been suggested that IL-8 secretion is linked to ER status, with ER positive cell lines producing no IL-8 whilst ER negative lines overexpress IL-8 (Freund et al., 2003). However, this study suggests that the opposite may be true for these particular immortal lines as IL-8 producing SKOV-3 is ER positive whilst OVCAR-5 is ER negative and secretes no IL-8. The low expression of VEGF in OVCAR-5 may be one reason why its cellular metabolism and proliferation was not affected by treatment with resveratrol or acetyl resveratrol. As these compounds are attenuating VEGF expression and this decrease is proposed to be responsible for the decline in SKOV-3's metabolism and growth. It can be hypothesised that if there is low VEGF secretion to begin with, then any reduction in secretion would not make a difference to cell growth. This would then raise the possibility that OVCAR-5 is not reliant on VEGF for growth and metastasis, whereas SKOV-3 depends more heavily on VEGF.

4.4.1 Resveratrol.

The dose and time dependency of VEGF attenuation by resveratrol in SKOV-3 cells correlates with its effect on metabolism and proliferation. Thus, it is suggested that VEGF may at least in part be responsible for the afore mentioned response. The relationship between VEGF suppression and enhanced apoptosis has been identified in a previous study of monolayers of normal endothelial cells treated with 10µM resveratrol for two days (Mikula-Pietrasik et al, 2012). Resveratrol at 0.5 –100µM has also been shown to reduce VEGF production in endometrial and ovarian cancer cell monolayer cultures after 24 hours exposure (Park et al., 2007, Dann et al., 2009). However, the upregulating effect of resveratrol on IL-8 secretion in both ovarian cancer cell lines has not been observed before. Previous studies on monolayer cultures of other cell lines have typically found that resveratrol suppresses IL-8 production (Aggarwal et al., 2004, Mikula-Pietrasik et al., 2012). However, Lee et al., 2000, observed that paclitaxel treatment induced IL-8 secretion in ovarian cancer; therefore it is possible that resveratrol is exerting this same effect in these particular cell lines. This raises the question of whether these cell lines are beginning to compensate for the reduction of VEGF, as IL-8 has been implicated in the tumorigenic and metastatic potential of other cancers (Xie, 2001). The use of 3D cell cultures in this study may represent likely *in vivo* responses more accurately. Therefore, I cautiously suggest that prolonged exposure to doses of at least 30µM resveratrol would be effective in restricting ovarian cancer tumour growth and metastasis (this of course would be dependent on the cell type of the origin of the cancer). This is a higher concentration and much longer exposure time than suggested in previous reports and is possibly due to the different cell culturing method used in this study.

4.4.2 Acetyl resveratrol.

As mentioned previously acetyl resveratrol was investigated due to the possibility that it might be more bioavailable than resveratrol (Liang et al, 2013). However, very little is known about the anticarcinogenic properties of acetyl resveratrol and, to the best of my knowledge, this is the first study of its effects on VEGF and IL-8 secretion. Its effect on both VEGF and IL-8 secretion were very similar to that of resveratrol, thus once again it is

suggested that prolonged exposure to 30µM acetyl resveratrol may attenuate metastasis and tumour growth in certain ovarian cancer types.

4.4.3 EGCG.

Studies have shown that EGCG is able to attenuate VEGF secretion in different cell types. In normal endothelial monolayer cell cultures EGCG downregulates VEGF binding and production at low concentrations, thereby inhibiting cell proliferation without inducing apoptosis (Kondo et al., 2002, Trompezinski et al., 2003). In several cancer cell monolayer cultures, such as endometrial, breast and colorectal cancer, higher doses of EGCG (55-100µM) decrease VEGF secretion, inhibit cell proliferation and induce apoptosis (Sartippour et al., 2002, Dann et al., 2009, Shimizu et al., 2010). Even in an ovarian cancer monolayer study using different cell lines to this study 20-40µM EGCG was found to decrease VEGF production, supress cell growth and increase apoptosis (Spinella et al, 2006). There appear to be few investigations into the link between EGCG and IL-8 yet there is evidence that EGCG decreases IL-8 secretion in homeostatic cells, although IL-8 production must first be stimulated with a pro-inflammatory cytokine (Chen et al., 2002, Trompezinski et al., 2003). However, in correlation with cellular metabolism and growth results in this study VEGF and IL-8 secretion is not affected in either cell line by EGCG treatment.

4.4.4 Resveratrol and EGCG combination.

Unfortunately once again no synergistic effect was evident between resveratrol and EGCG in regards to VEGF and IL-8 secretion. This was somewhat unexpected as previous investigations have revealed that resveratrol and EGCG have a synergistic effect in combination with other natural products (Suganuma et al., 1999, Conte et al., 2003). Furthermore, the polyphenols have been shown to affect multiple cancer growth pathways and some of their activity does not overlap (Fig. 4.9) (Hemalswarya and Doble, 2006).

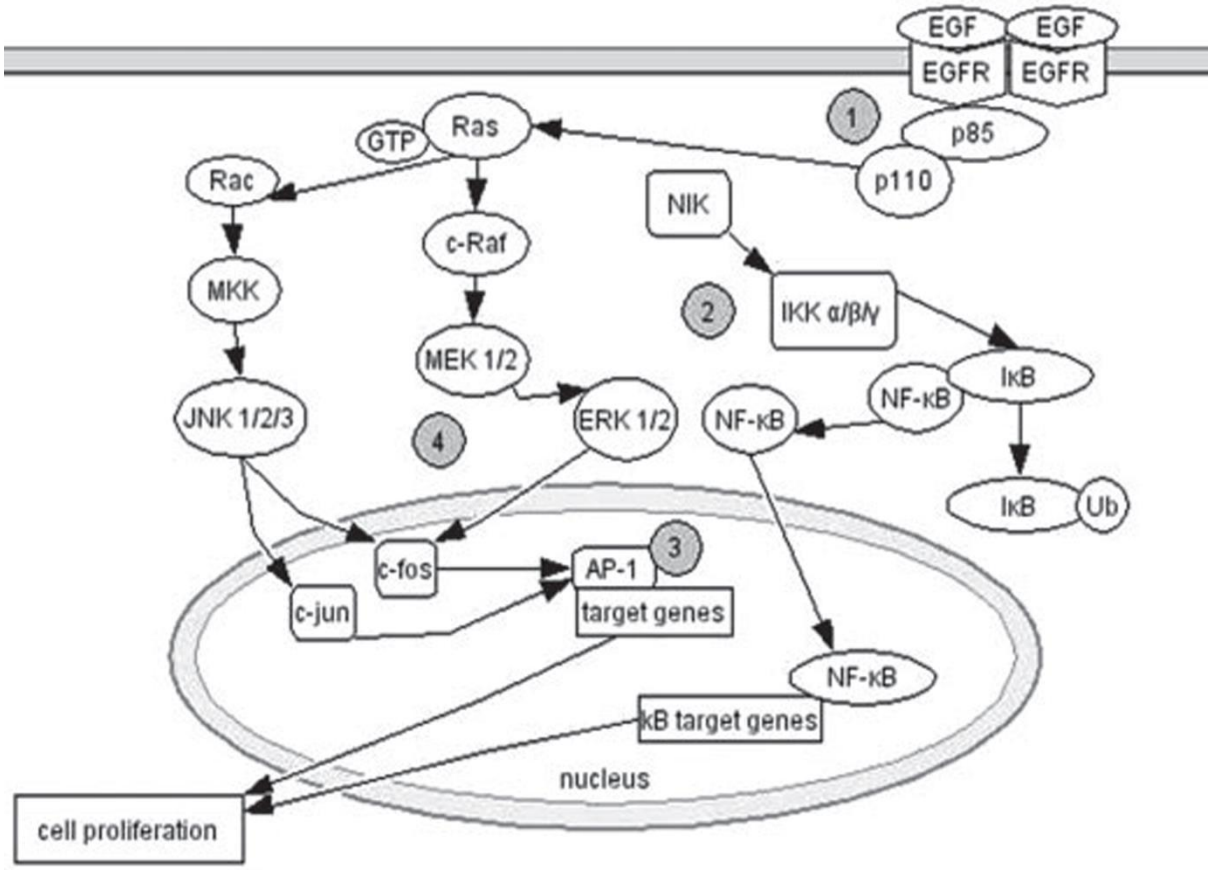

Figure 4.9 Proposed molecular targets for resveratrol and EGCG in cancer. 1 - resveratrol blocks EGFR. 2 - resveratrol and EGCG inhibit the NF-κB pathway. 3 - resveratrol and EGCG inhibit AP-1 pathway. 4 - EGCG blocks the MAPK signalling pathway. Image from Hemalswarya and Doble, 2006.

Chapter 5

Signalling Protein Expression.

5.1 Introduction.

The results presented in Chapter 4 suggest that VEGF is decreased by resveratrol and acetyl resveratrol in ovarian cancer cells grown in 3D cultures. The next stage in the investigation was to investigate the possible mechanisms that may be associated with VEGF suppression in spheroids/clusters of ovarian cancer cells. The signalling pathways that control cell proliferation, survival, and oncogenesis are of interest in research for the development of targeted cancer cell therapy (Rayet and Gelinas, 1999). One such pathway that appears to be prominent in cancer cell growth and survival is the NF-κB pathway. Since the discovery of NF-κB there has been much research into its transcriptional and biological functions, and the mechanisms that control its activity (Athar et al., 2009). The secretion of VEGF and IL-8 have been linked to the activation of the NF-κB signalling pathway in many types of normal and cancerous cells (Birk et al., 2008, Novotny et al., 2008, Leychenko et al., 2011). Furthermore, binding sites for NF-κB on or near the promoter sequences of VEGF and IL-8 have been identified (Royds et al., 1998, Roebuck 1999).

5.1.1 Nuclear Factor-Kappa B (NF-κB).

5.1.1.1 Background.

The NF-κB proteins are a small family of inducible transcription factors that include RelA (p65), RelB, p-50 and p52 (Rayet and Gelinas, 1999). Nearly all of this family can form homo- or heterodimers; it is the p50-RelA heterodimer that is called NF- κB (Rayet and Gelinas, 1999). This highly regulated dimer is a positive mediator of T- and B- cell development, survival and proliferation (Jost and Ruland, 2007). It is involved in the regulation of a variety of cellular processes such as inflammation, immune and stress responses, cell growth regulation, as well as oncogenesis and cell transformation (Athar et al., 2009). In the inactive state NF-κB is bound and sterically blocked by the inhibitor protein IkBα in the cytoplasm (Pervais, 2004). In the active state, which is stimulated by

nutrients, growth factors, cytokines and chemokines, several upstream kinases such as mitogen-activated protein kinases (MAPK) phosphorylate IκB kinase. This in turn phosphorylates IκBα which is then polyubiquitinated and subsequently degraded (Kraft et al., 2009). Thus, NF-κB is released, allowing nuclear translocation of the active dimer and the activation of gene transcription (Jost and Ruland, 2007). Defects in NF-κB lead to increased apoptosis due to its regulation of anti-apoptotic genes. Incorrect regulation of the transcription factor has been associated with inflammatory and autoimmune diseases, septic shock, viral infection and cancer (Shukla and Singh, 2011).

5.1.1.2 NF-κB and Cancer.

Since the identification of NF-κB it has been suspected to be involved in the development of some cancers due to its similarity to the oncogene v-Rel (Karin, 2009). Thus far the NF-κB pathway has been shown to be involved in the survival of numerous cancers such as skin, prostate and lung cancer (Shukla and Singh, 2011). Many of these cancers display constitutively high levels of NF-κB due to hyperactivation of the signalling pathway or inactivating mutations in IκBα (Rayet and Gelinas, 1999). Constitutive activation is a critical pathogenic factor in lymphoma and aberrant activation correlates with a poor clinical outcome in diffuse large B-cell lymphoma (Jost and Ruland, 2007). Persistent activation of NF-κB has also been identified in breast, stomach and thyroid cancers (Rayet and Gelinas, 1999). However, oncogenic mutations within NF-κB appear to be rare (Karin, 2009) and chromosomal aberrations involving NF-κB are infrequently found in lymphomas and leukaemias (Rayet and Gelinas, 1999). It has been hypothesised that sustained activation of NF-κB is necessary for growth, survival and tumourigenicity of many different tumour types through its homeostatic regulation of inflammatory responses and anti-apoptotic genes (Karin, 2009). As an example in multiple myeloma cells, supressed NF-κB resulted in decreased anti-apoptotic genes cyclin D1, cIAP-2, XIAP, survivin, Bcl2, BclxL, Bfl-1/A1, TRAF2 and Akt (Althar et al., 2009) and inflammatory components have been identified in the microenvironment of tumours that are not epidemiologically related to inflammation (Karin, 2009). Hypoxia has also been identified as an inducer of the NF-κB pathway (Schmidt et al., 2007). Ascites induced by the metastatic stages of ovarian cancer is a hypoxic condition; therefore, it is proposed that the NF-κB pathway is upregulated and integral to the survival of ovarian cancer cells.

5.1.1.3 NF-κB and Resveratrol.

In previous studies it has been reported that resveratrol attenuates the NF-κB pathway in breast and pancreatic cancers, as well as in multiple myeloma and acute myeloid leukaemia (Pervaiz, 2004, Athar et al., 2009, Goswami and Das, 2009). To date, the targets of resveratrol have been identified as IκBα, where it blocks the phosphorylation and therefore degradation of the inhibitor (Pervais, 2004), IκB kinase, suppression of IκB kinase phosphorylation again results in inhibited degradation of I IκBα (Bianchini and Vainio, 2003), and perhaps even attenuation of NF-κB itself (Athar et al., 2009).

5.1.2 Aims of Chapter 5.

This chapter investigates the effect of resveratrol and acetyl resveratrol on the signalling proteins pIκBα, NF-κB and pNF-κB. This will demonstrate if the NF-κB signalling pathway plays a role in resveratrol and acetyl resveratrol's ability to reduce VEGF secretion.

5.2 Methods and Materials.

Experimental 3D spheroid cell cultures -See Chapter 2 for full method and materials.

BCA Protein Assay –See Chapter 2 for full method and materials.

Western Blotting –See Chapter 2 for full method and materials.

Frozen Sectioning and Immunofluorescence –See Chapter 2 for full method and materials.

5.3 Results.

5.3.1 Six Day NF-κB inhibitor Treatment Effects on Cellular Metabolism and Proliferation.

To investigate if the NF-κB protein is associated with cell viability and growth a specific NF-κB inhibitor, Bay 1170805, was employed. Cellular metabolism of SKOV-3 spheroids/clusters was reduced in a dose dependent fashion by treatment with the NF-κB inhibitor for 6 days. The lowest concentration of Bay 117085 tested, 10µM, resulted in a 35% decrease in metabolic rate. Concentrations of 20µM and 30µM inhibitor treatment reduced metabolism by 62% and 79% respectively (Fig. 5.1 a). Bay 117085 also caused a dose dependent decrease in SKOV-3 cell growth by as much as 52% at 30 µM (Fig. 5.1 c).

The cell growth and metabolism of the OVCAR-5 cell line was also dose dependently adversely affected by NF-κB inhibition, albeit not as strongly as the SKOV-3 cell line (Fig. 5.1). Metabolic rate was decreased by 40% and 24% with 20µM and 30µM Bay 117085, respectively (Fig. 5.1 b). OVCAR-5 cell growth also declined by 18% when treated with 20µM of inhibitor and by 28% when treated with 30µM of inhibitor (Fig. 5.1 d).

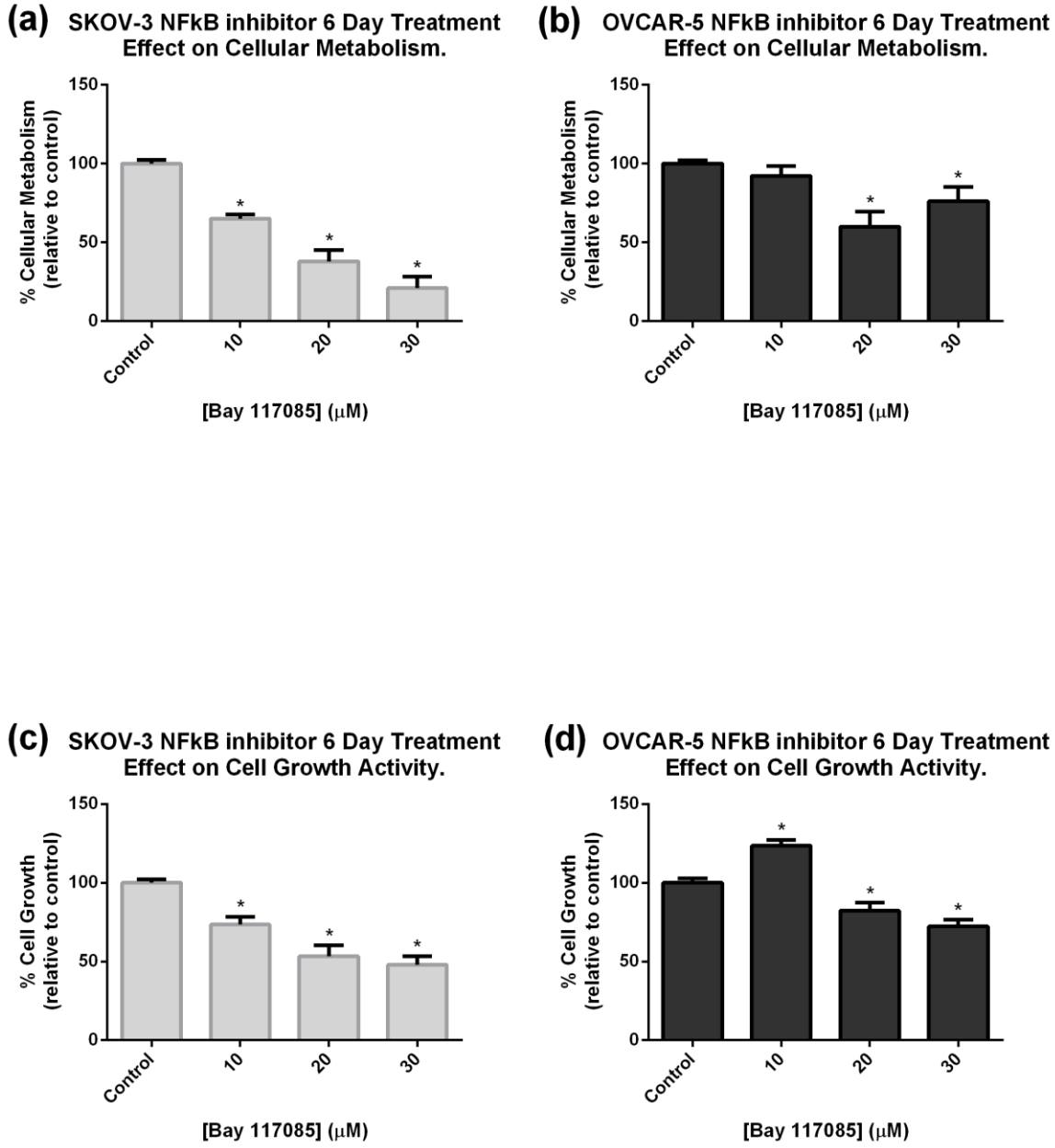

Figure 5.1. Relative cellular metabolism (as determined by Alamar Blue assay) and growth activity (as determined by a crystal violet assay) of SKOV-3 (a, c) and OVCAR-5 (b, d) spheroids/clusters treated with various concentrations of NF-κB inhibitor Bay 117085 for 6 days. Data are from four independent experiments carried out in triplicate ± SEM. * denotes statistical significance (t-test, $P < 0.05$) relative to the control.

5.3.2 Protein expression.

Western blotting was performed to determine the expression of three signalling proteins and one cell cycle protein in SKOV-3 and OVCAR-5 spheroids/clusters exposed for 6 days to either resveratrol or acetyl resveratrol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference housekeeping protein, due to its relatively constant expression in the cell lines being used, despite changing cell conditions. This was quantitatively assessed via the density of the GAPDH bands (Fig.5.2 e, 5.5 d, 5.8 d, 5.11 e, 5.14), and was therefore deemed most appropriate for these analyses. Western blotting of alternative housekeeping proteins was carried out but none were as suitable as a housekeeping protein (See Appendix).

5.3.2.1 Protein Expression in SKOV-3 spheroids/clusters Treated for 6 days with Resveratrol.

In the SKOV-3 cell line, NF-κB and pNF-κB showed a dose dependant decrease after 6 days treatment with resveratrol (Fig. 5.2 b, c). The proportion of phosphorylated NF-κB to non-phosphorylated NF-κB was between 25 – 28% after 6 days resveratrol treatment. pIκBα and PCNA were unaffected by resveratrol treatment (Fig. 5.2 a, d). Immunofluorescence showed that NF-κB (stained red) was located in the cytoplasm and the nuclei (stained blue) of SKOV-3 cells grown as spheroids/clusters (Fig. 5.3). After 6 days treatment with 20μM and 30μM resveratrol there was a noticeable reduction of NF-κB located inside the nuclei and an overall reduction of NF-κB expression (Fig. 5.3). Z-stack imaging (not shown) was used to confirm that pNF-κB was also located inside nuclei as it was harder to discern than NF-κB (Fig. 5.3, Fig. 5.4). There was also a reduction of overall pNF-κB expression and nuclear location after 6 days treatment with 30μM resveratrol (Fig. 5.4).

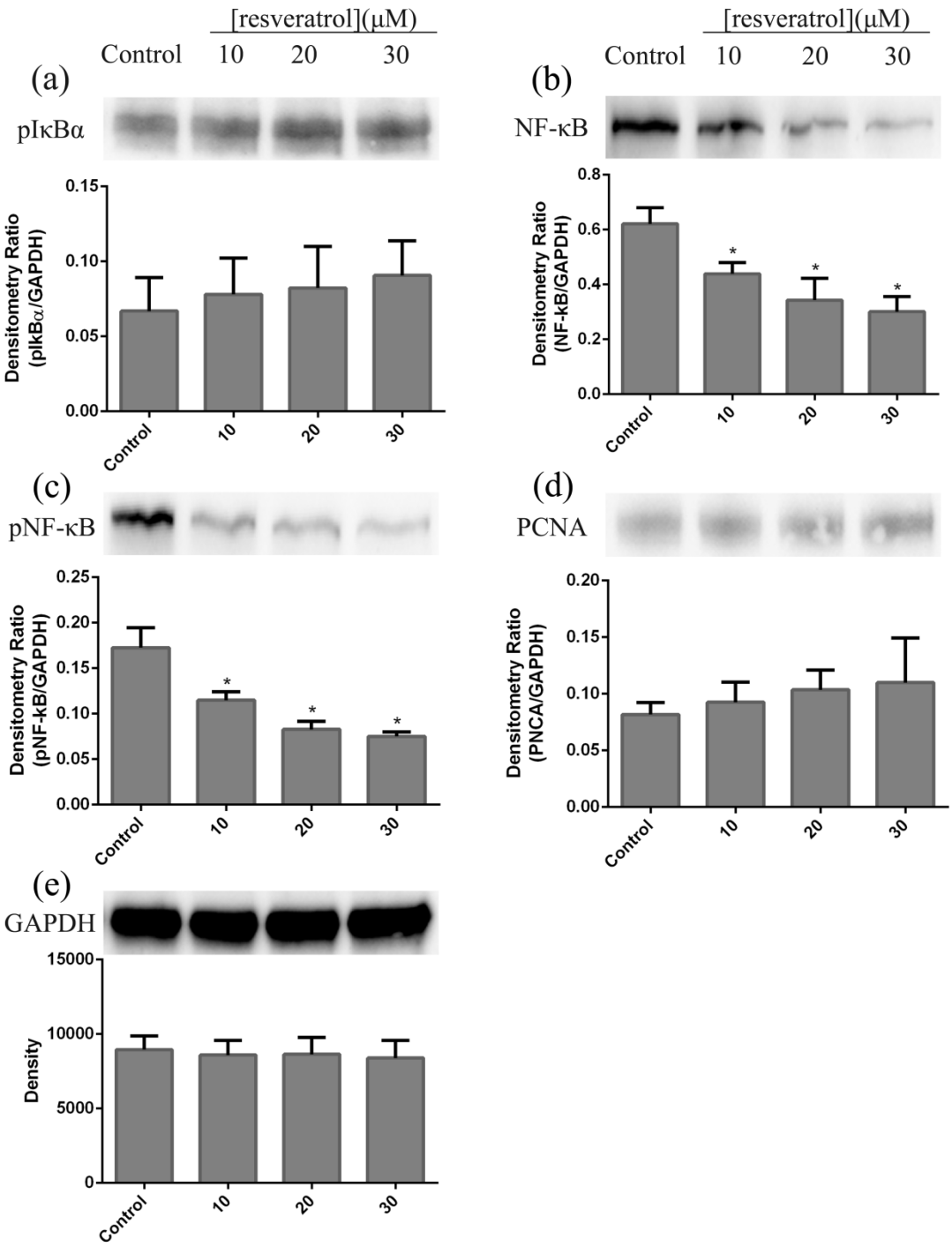

Figure 5.2. Effects of resveratrol treatment for 6 days on SKOV-3 spheroids/clusters. Western blot and densitometry ratios of (a) pIκBα (b) NF-κB (c) pNF-κB (d) PCNA (e) GAPDH. Western blot images are representative of three experiments carried out in duplicate. The glycolytic enzyme GAPDH was used as a reference protein and 10 μg of protein per well was loaded for each sample. Densitometry ratios are relative to GAPDH, whose expression did not change after treatment (e). Results are representative of three independent experiments analysed in duplicate.

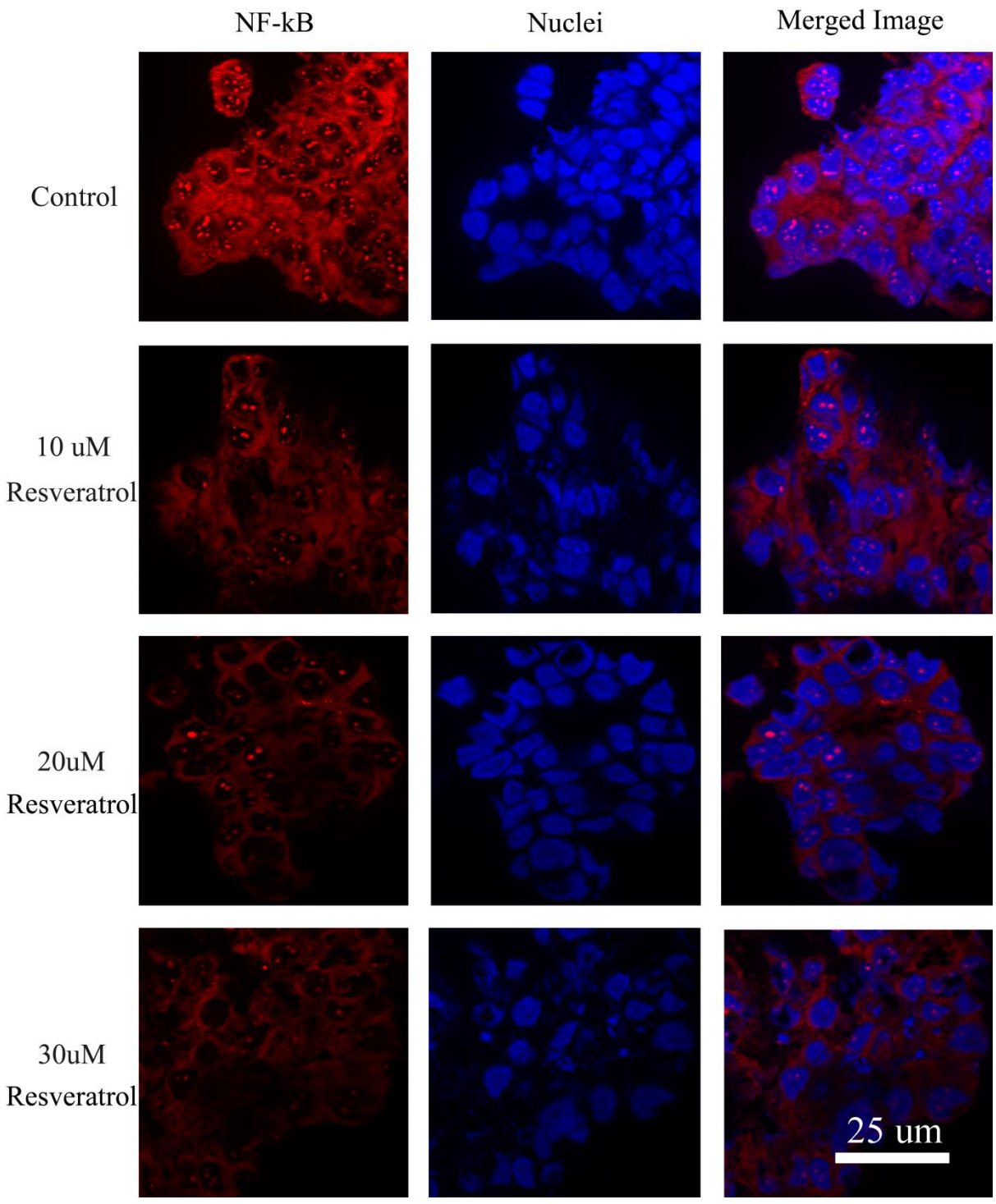

Figure 5.3. Immunofluorescent images of NF-κB in frozen cut sections of SKOV-3 spheroids/clusters treated with resveratrol for 6 days. NF-κB was stained red (left column) and nuclei were stained blue (middle column).

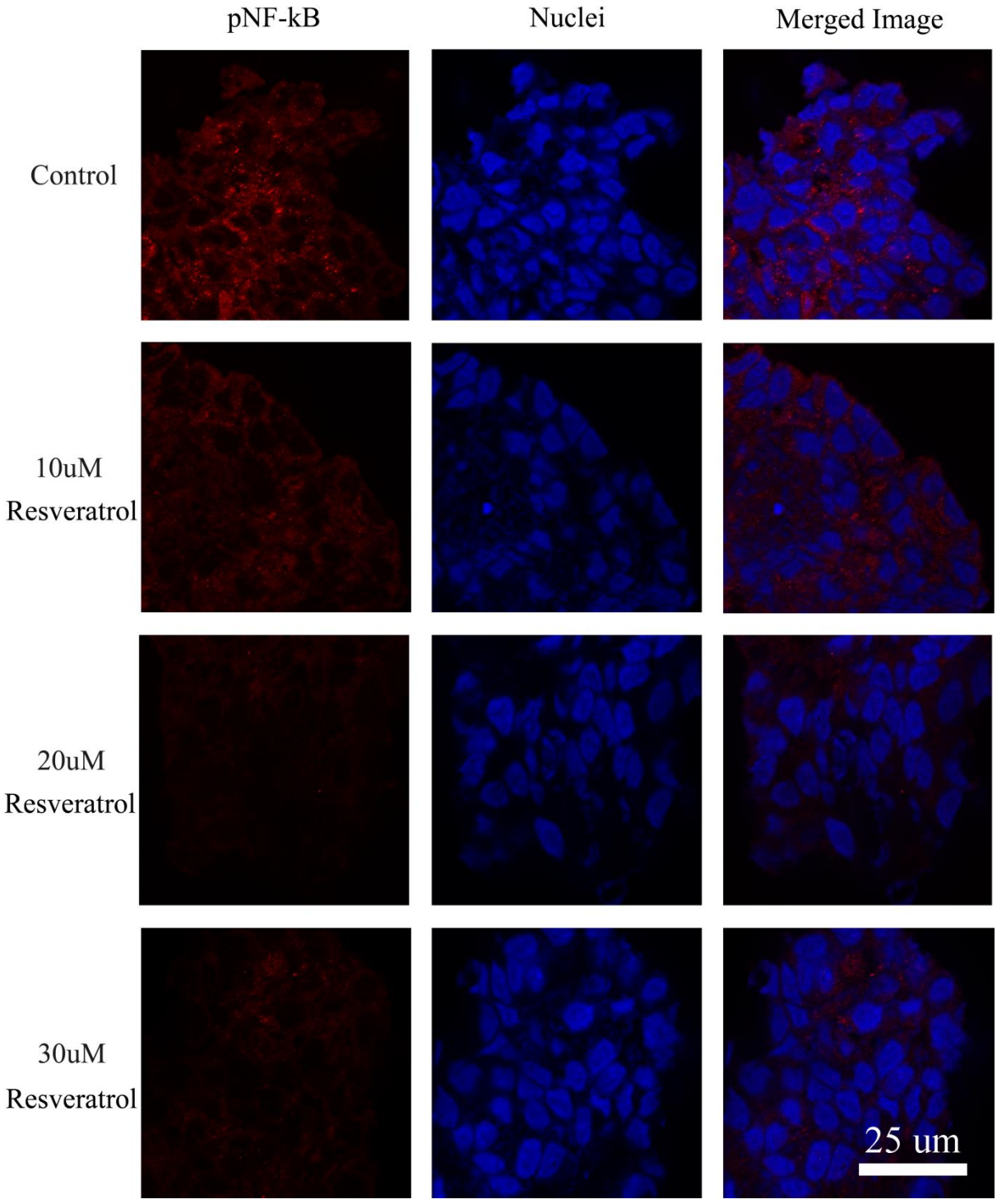

Figure 5.4. Immunofluorescent images of pNF-κB in frozen cut sections of SKOV-3 spheroids/clusters treated with resveratrol for 6 days. pNF-κB was stained red (left column) and nuclei were stained blue (middle column).

5.3.2.2 Protein Expression in OVCAR-5 spheroids/clusters Treated for 6 days with Resveratrol.

The trend of dose dependent decrease of NF-κB and pNF-κB after 6 days treatment with resveratrol was also evident in the OVCAR-5 cell line (Fig. 5.5). There was approximately 5 times more NF-κB present in the OVCAR-5 cell line compared to SKOV-3 (Fig. 5.2, Fig. 5.5). Furthermore, the proportion of phosphorylated NF-κB to non-phosphorylated NF-κB expressed by OVCAR-5 was much greater than in SKOV-3, on average it was around 70% after resveratrol treatment. Immunofluorescent images of the OVCAR-5 spheroids/clusters showed almost no NF-κB or pNF-κB located in the nuclei (Fig. 5.6, Fig. 5.7). The expression of NF-κB and pNF-κB did not appear to change after 6 days of resveratrol treatment (Fig. 5.6, Fig. 5.7).

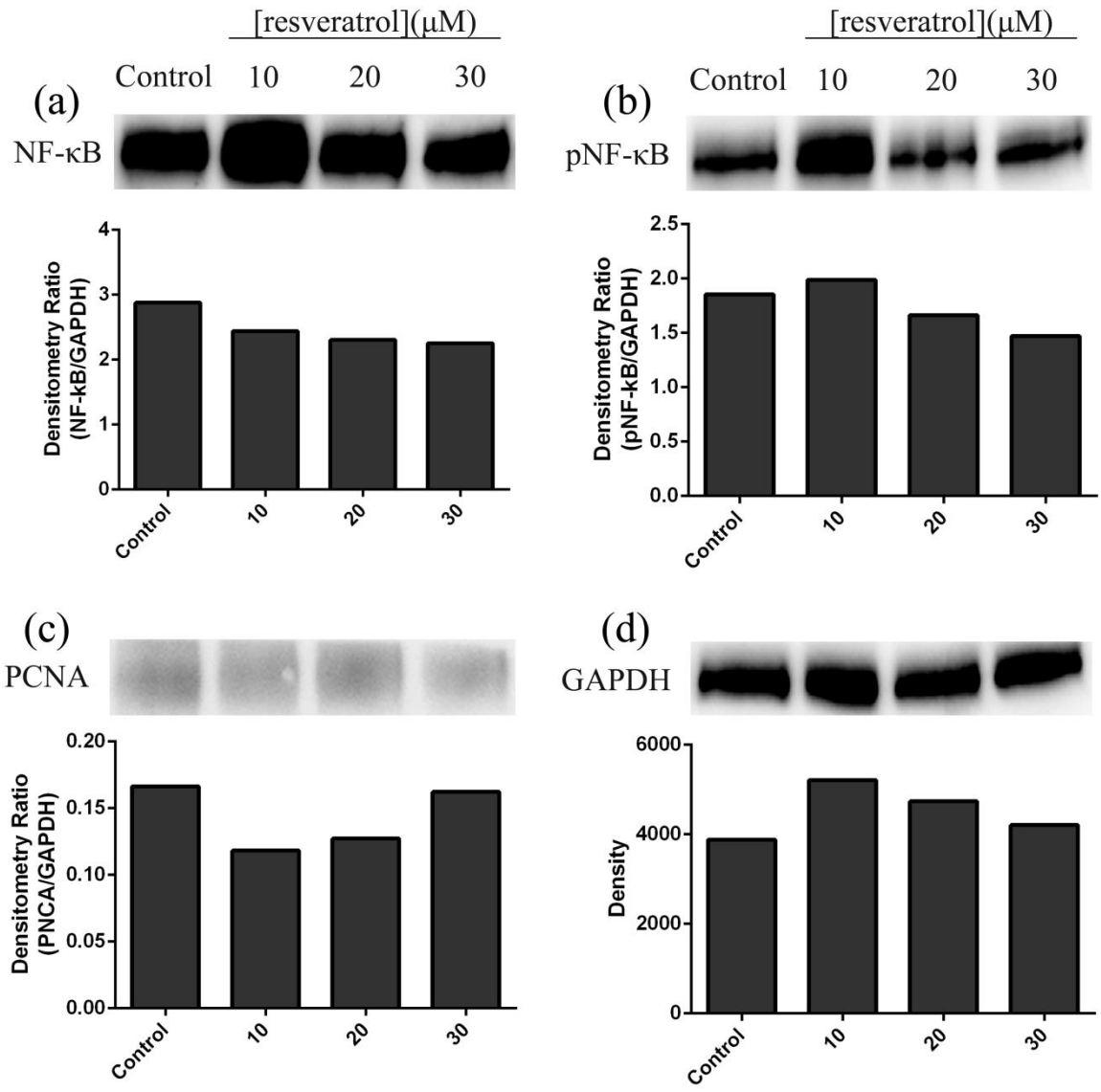

Figure 5.5. Effects of resveratrol treatment for 6 days on OVCAR-5 spheroids/clusters. Western blot and densitometry ratios of (a) NF-κB (b) pNF-κB (c) PCNA (d) GAPDH. Western blot images are representative of one experiment carried out in duplicate. The glycolytic enzyme GAPDH was used as a reference protein and 10 μg of protein per well was loaded for each sample. Densitometry ratios are relative to GAPDH, whose expression did not change after treatment (d). Results are representative of one experiment analysed in duplicate.

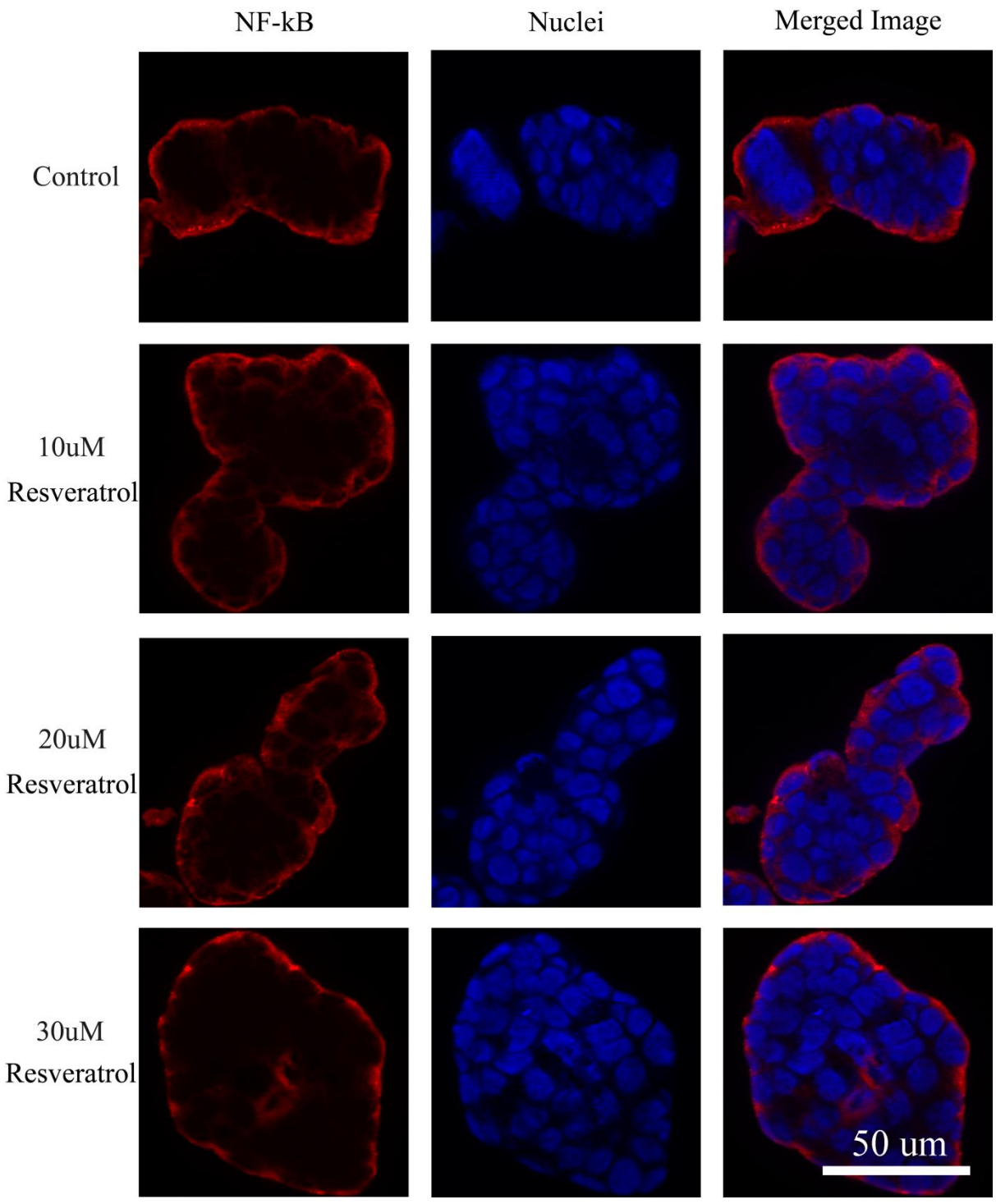

Figure 5.6. Immunofluorescent images of NF-κB in OVCAR-5 spheroids/clusters treated with resveratrol for 6 days. NF-κB was stained red (left column) and nuclei were stained blue (right column).

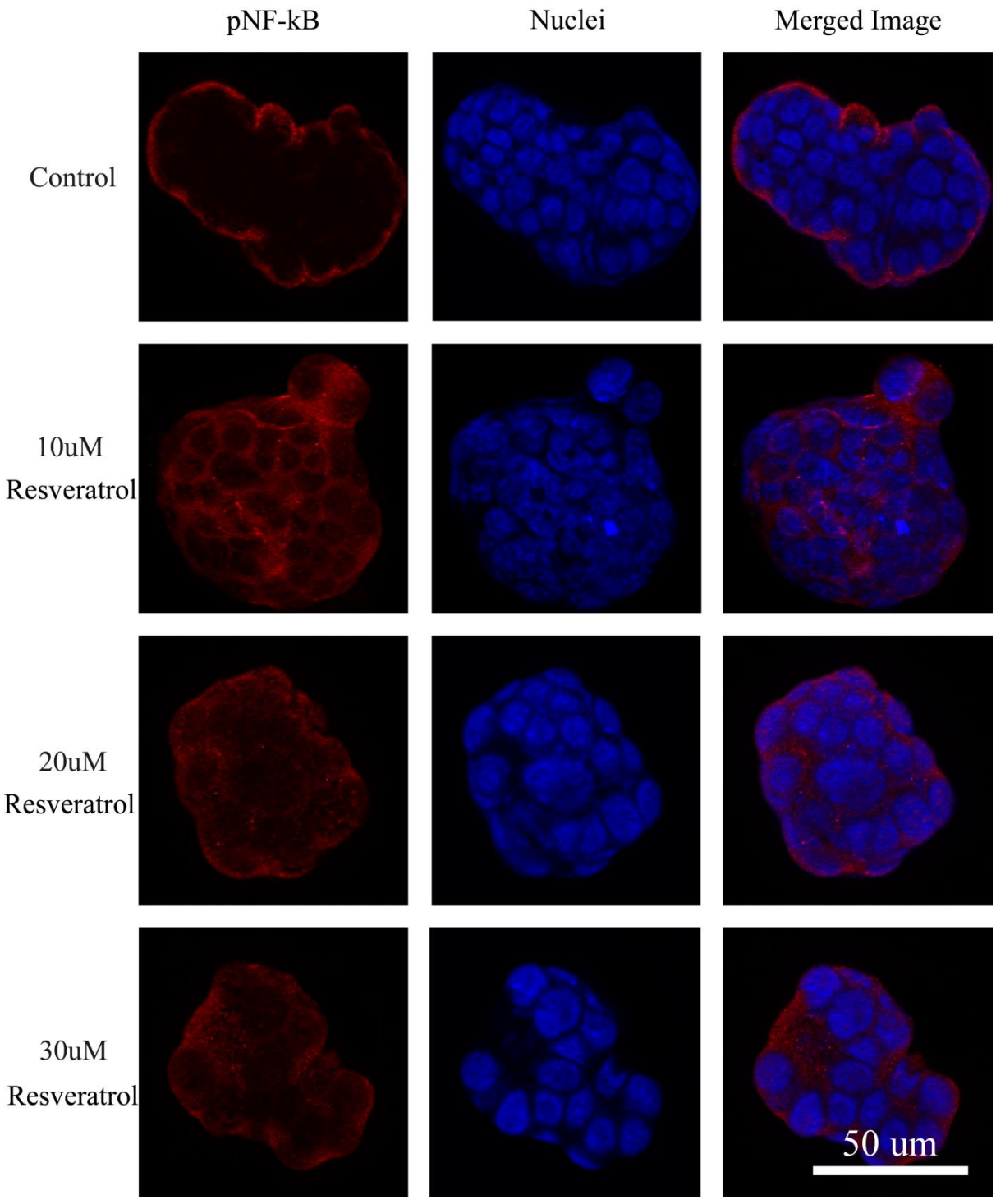

Figure 5.7. Immunofluorescent images of pNF-κB in OVCAR-5 spheroids/clusters treated with resveratrol for 6 days. pNF-κB was stained red (left column) and nuclei were stained blue (right column).

5.3.2.3 Protein Expression in SKOV-3 spheroids/clusters Treated for 6 days with Acetyl resveratrol.

pIκBα expression showed a dose dependant increase after 6 days treatment with acetyl resveratrol (Fig. 5.8 a). NF-κB and pNF-κB were not affected by the treatment (Fig. 5.8 b, c). The proportion of phosphorylation of NF-κB after 6 days acetyl resveratrol treatment was between 38 – 43%. Immunofluorescence showed that NF-κB and pNF-κB were located in the nuclei and cytoplasm, and their expression did not visibly alter after acetyl resveratrol treatment (Fig. 5.9, Fig. 5.10).

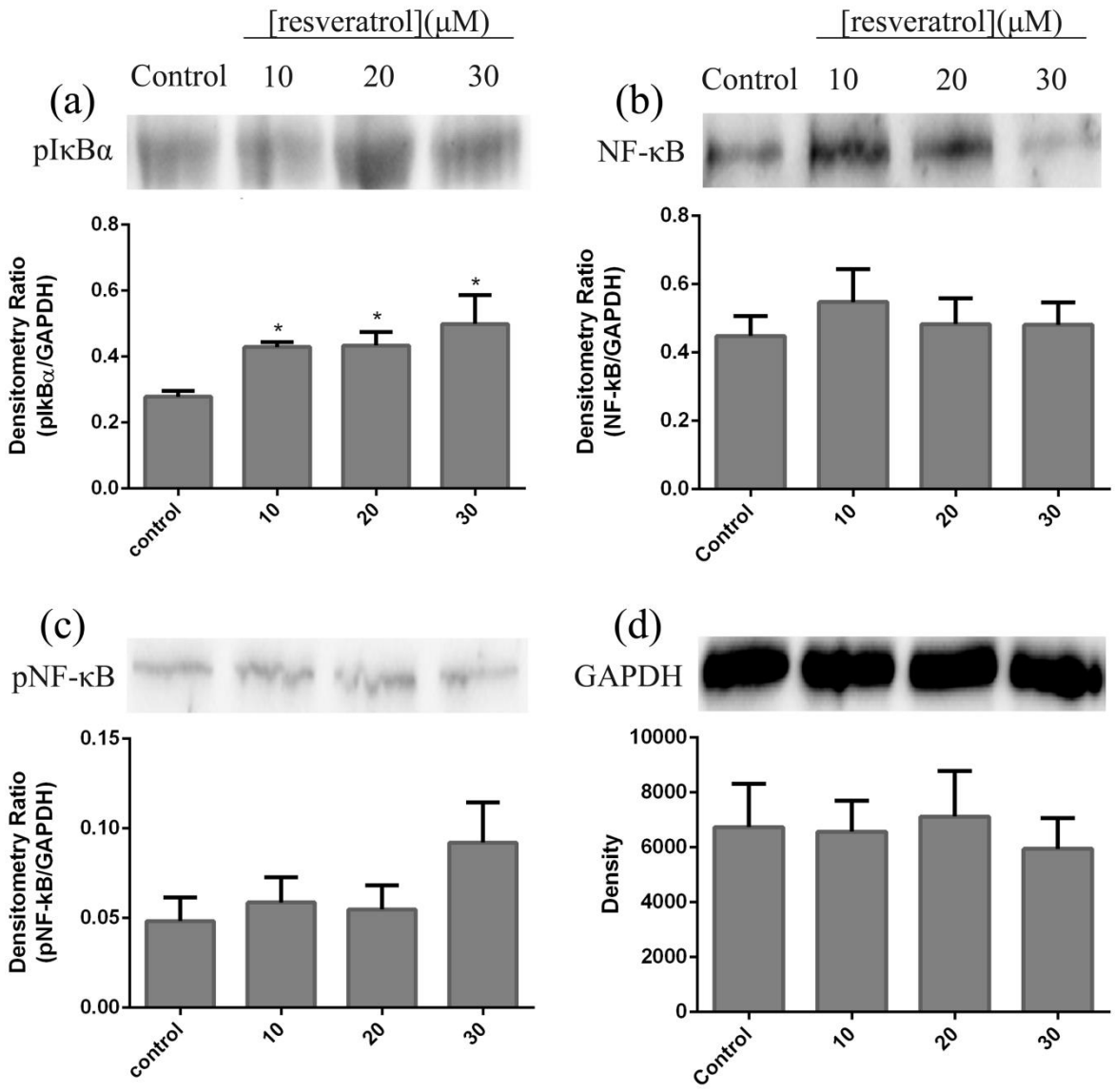

Figure 5.8. Effects of acetyl resveratrol treatment for 6 days on SKOV-3 spheroids/clusters. Western blot and densitometry ratios of (a) pIκBα (b) NF-κB (c) pNF-κB (d) GAPDH. Western blot images are representative of three experiments carried out in duplicate. The glycolytic enzyme GAPDH was used as a reference protein and 10 μg of protein per well was loaded for each sample. Densitometry ratios are relative to GAPDH, whose expression did not change after treatment (d). Results are representative of three independent experiments analysed in duplicate.

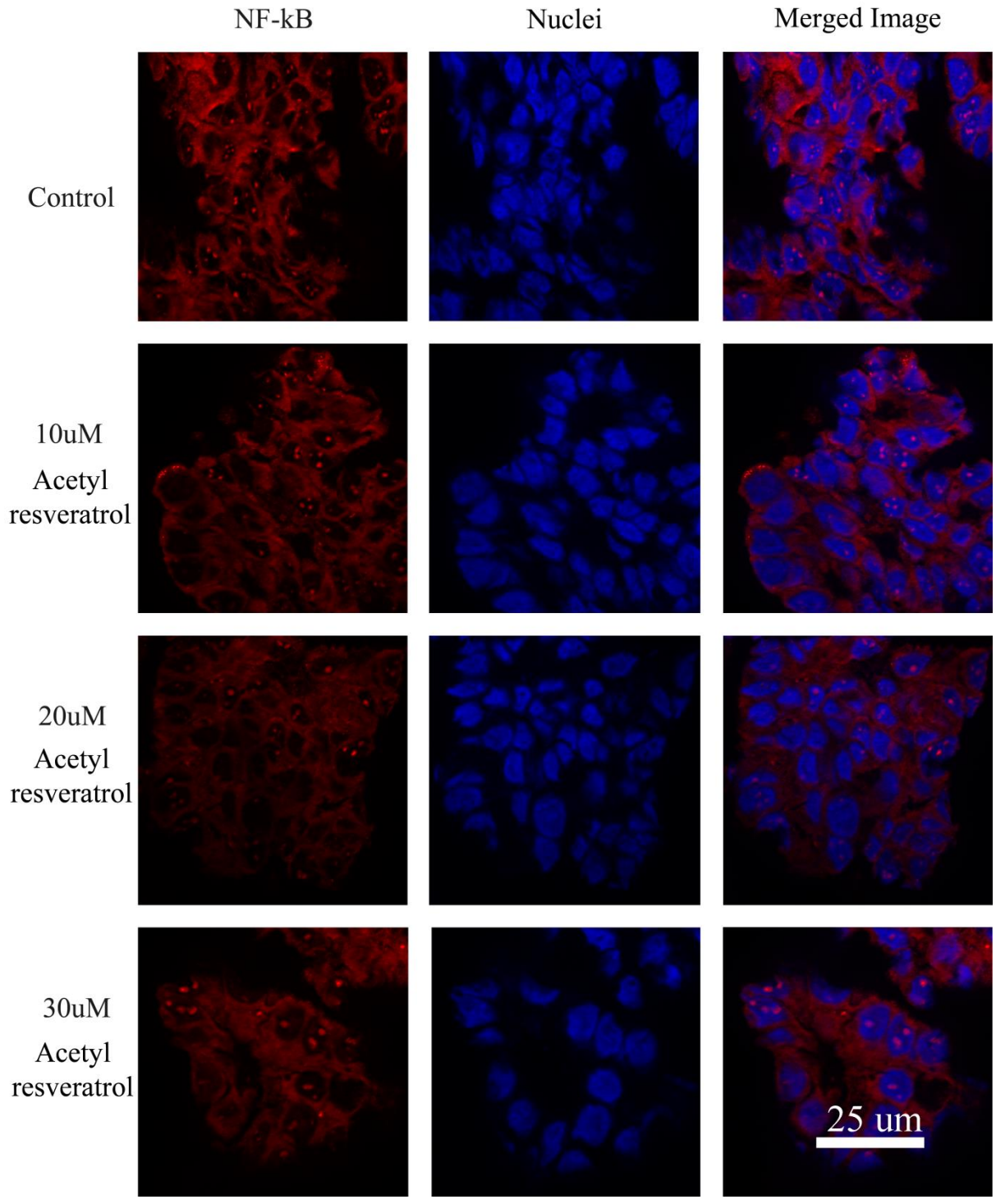

Figure 5.9. Immunofluorescent images of NF-κB in frozen cut sections of SKOV-3 spheroids/clusters treated with acetyl resveratrol for 6 days. NF-κB was stained red (left column) and nuclei were stained blue (middle column).

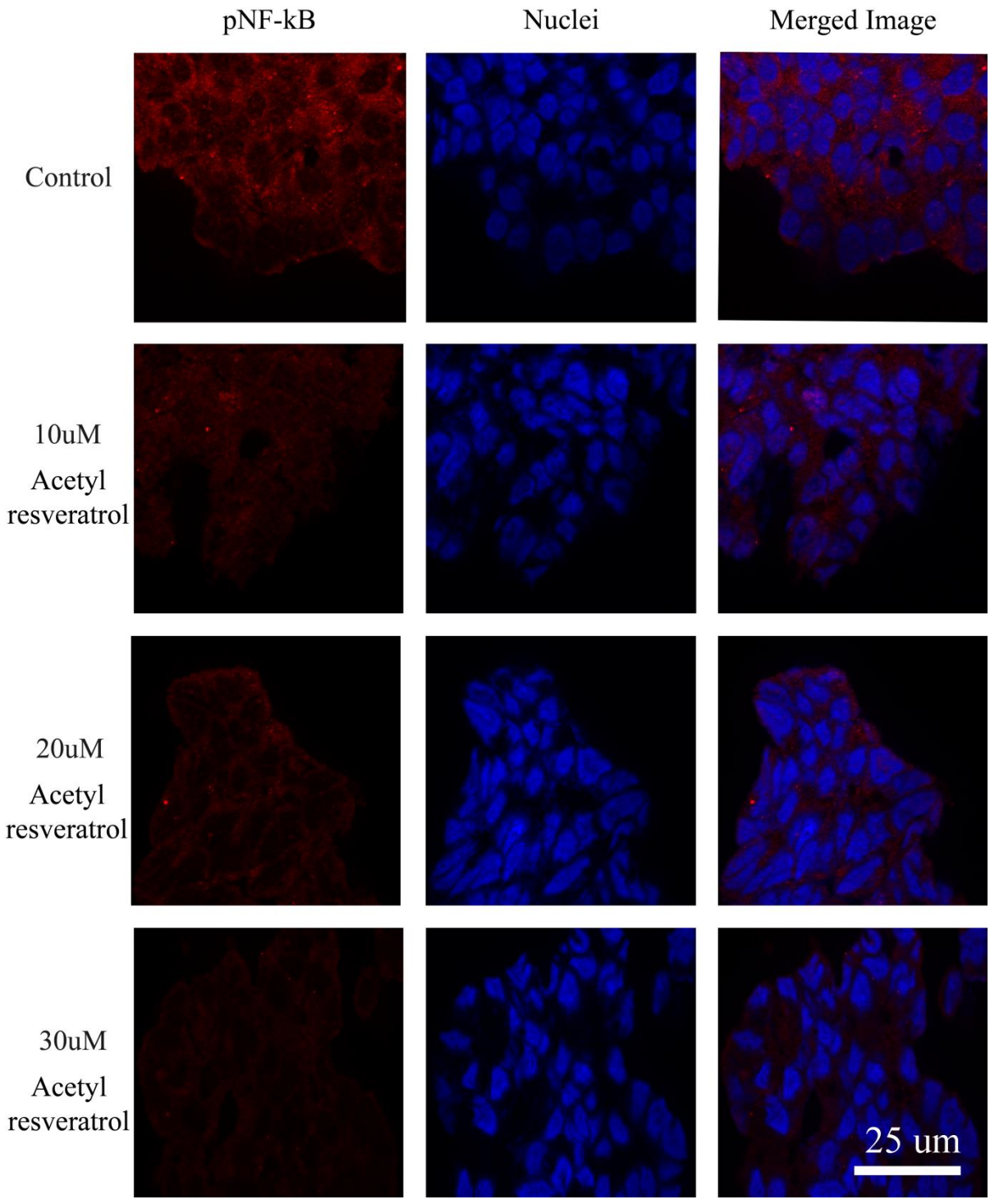

Figure 5.10. Immunofluorescent images of pNF-κB in frozen cut sections of SKOV-3 spheroids/clusters treated with acetyl resveratrol for 6 days. pNF-κB was stained red (left column) and nuclei were stained blue (middle column).

5.3.2.4 Protein Expression in OVCAR-5 spheroids/clusters Treated for 6 days with Acetyl resveratrol.

A trend of dose dependent decrease of NF-κB after 6 days treatment with acetyl resveratrol was evident in the OVCAR-5 cell line (Fig. 5.11). There was approximately 6 times more NF-κB present in the OVCAR-5 cell line compared to SKOV-3 (Fig. 5.8, Fig. 5.11). Furthermore, the proportion of phosphorylated NF-κB to non-phosphorylated NF-κB expressed by OVCAR-5 was again much greater than in SKOV-3. Immunofluorescent images of the OVCAR-5 spheroids/clusters showed almost no NF-κB or pNF-κB located in the nuclei (Fig. 5.12, Fig. 5.13). The expression of NF-κB and pNF-κB did not appear to change after 6 days of acetyl resveratrol treatment (Fig. 5.12, Fig. 5.13).

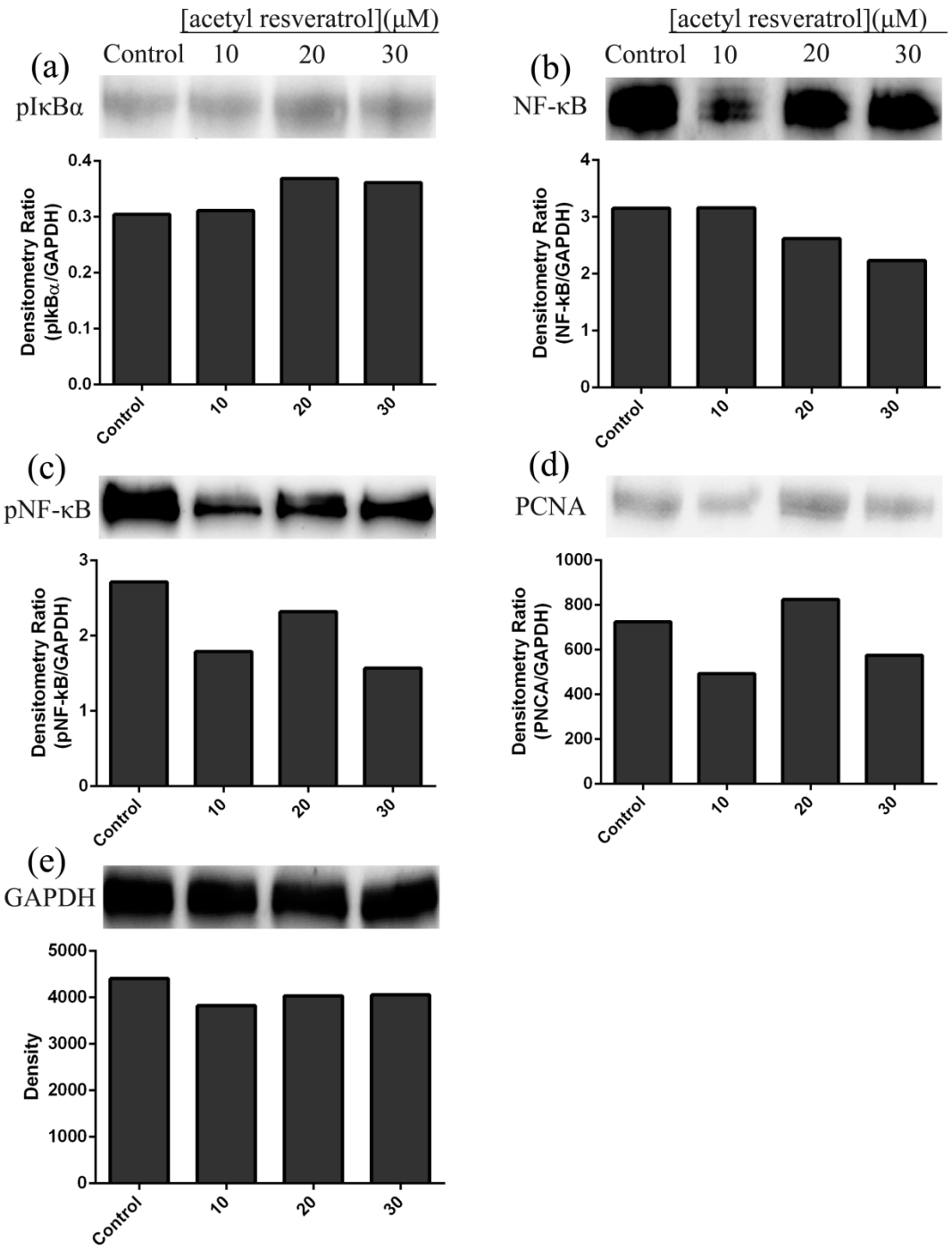

Figure 5.11. Effects of acetyl resveratrol treatment for 6 days on OVCAR-5 spheroids/clusters. Western blot and densitometry ratios of (a) pIκBα (b) NF-κB (c) pNF-κB (d) PCNA (e) GAPDH. Western blot images are representative of one experiment carried out in duplicate. The glycolytic enzyme GAPDH was used as a reference protein and 10 μg of protein per well was loaded for each sample. Densitometry ratios are relative to GAPDH, whose expression did not change after treatment (e). Results are representative of one experiment analysed in duplicate.

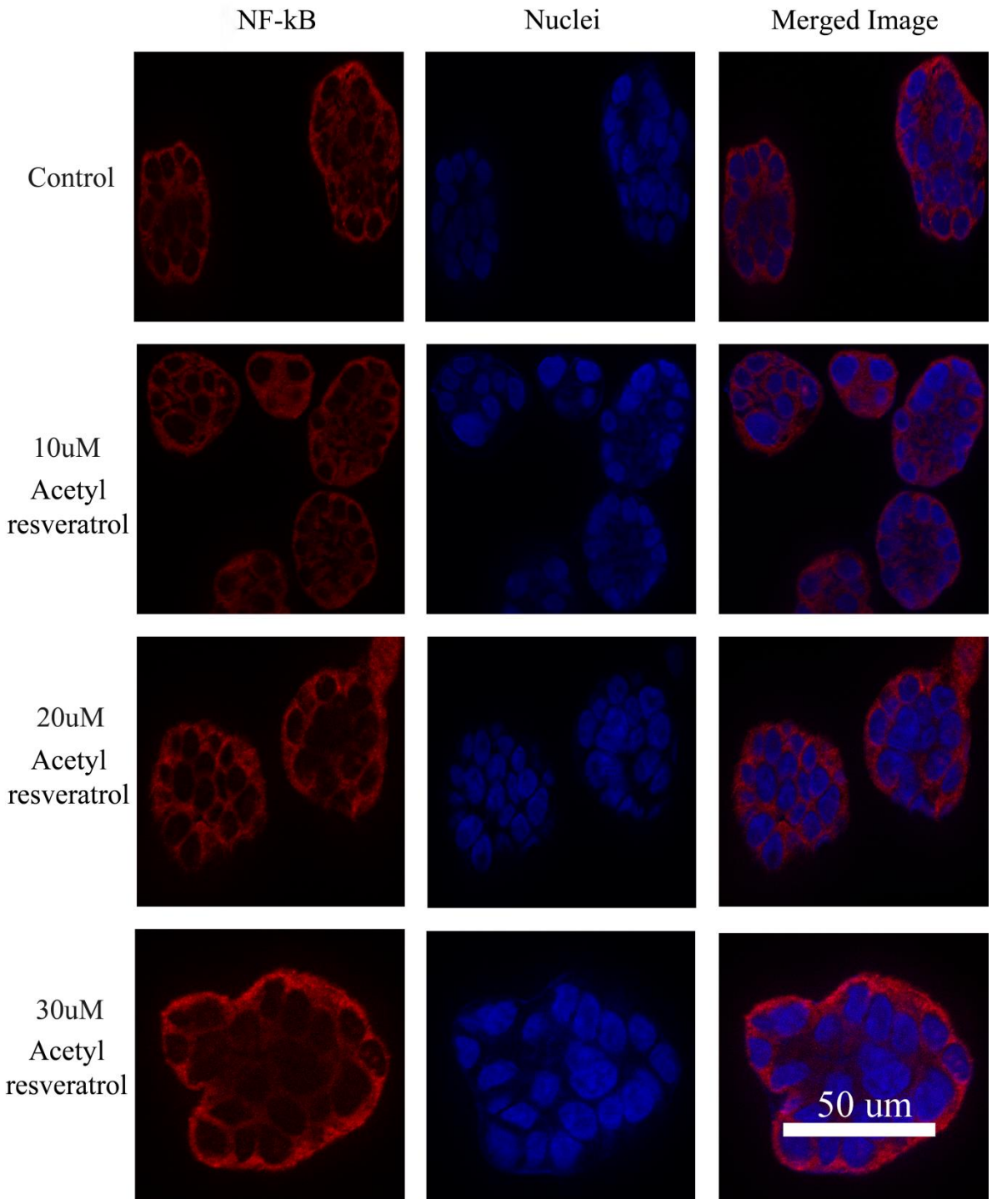

Figure 5.12. Immunofluorescent images of NF-κB in OVCAR-5 spheroids/clusters treated with acetyl resveratrol for 6 days. NF-κB was stained red (left column) and nuclei were stained blue (right column).

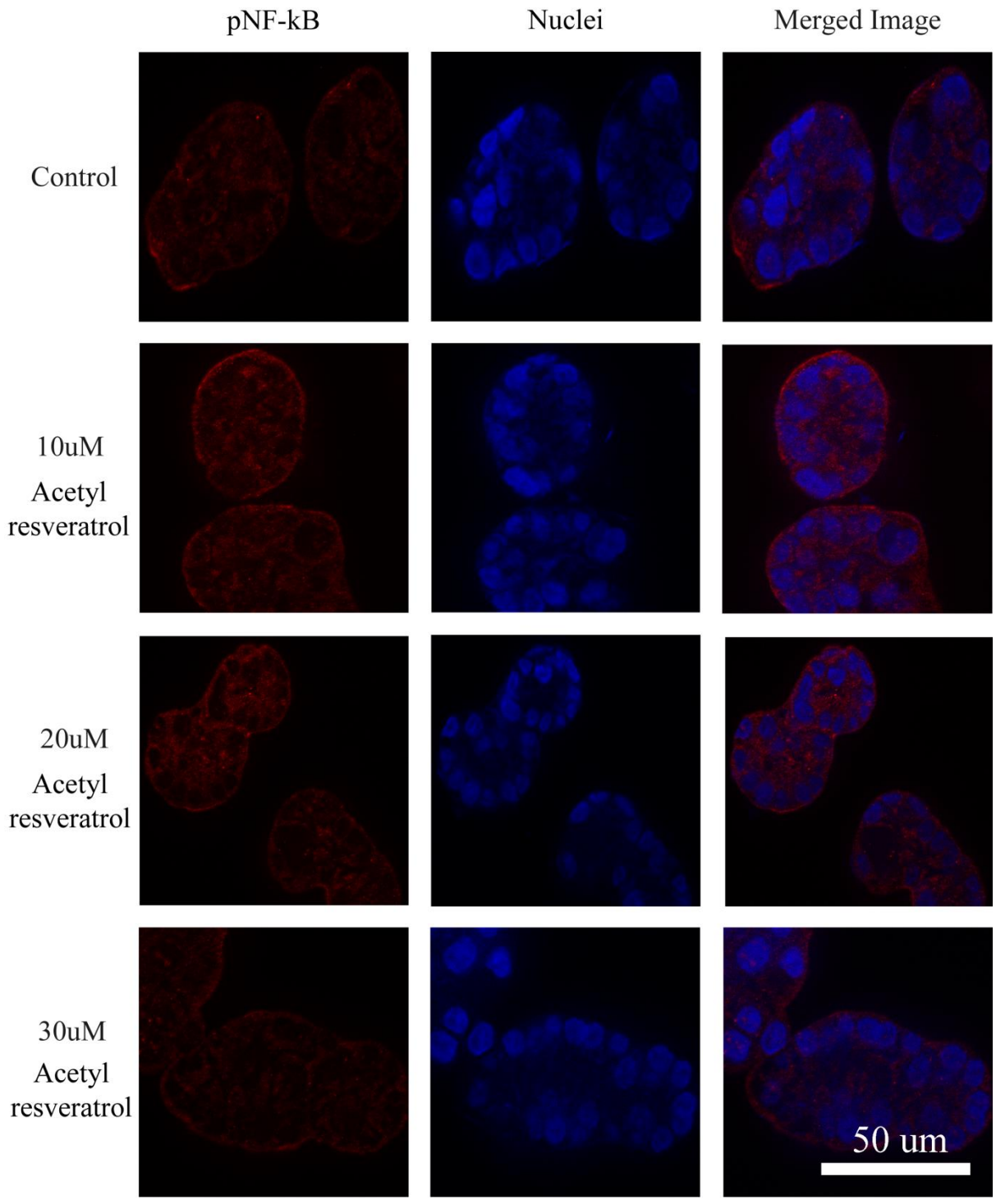

Figure 5.13. Immunofluorescent images of pNF-κB in OVCAR-5 spheroids/clusters treated with acetyl resveratrol for 6 days. pNF-κB was stained red (left column) and nuclei were stained blue (right column).

5.3.2.5 Protein Expression in SKOV-3 spheroids/clusters Treated for 4 days with Resveratrol.

Western blot analysis showed that treatment of SKOV-3 spheroids/clusters for 4 days with resveratrol did not significantly affect any of the proteins of interest (Fig. 5.14). The overall expression of pIκBα was at least two fold greater than expression in SKOV-3 spheroids/clusters treated for 6 days with either resveratrol or acetyl resveratrol (Fig. 5.2, Fig. 5.8, Fig. 5.14).

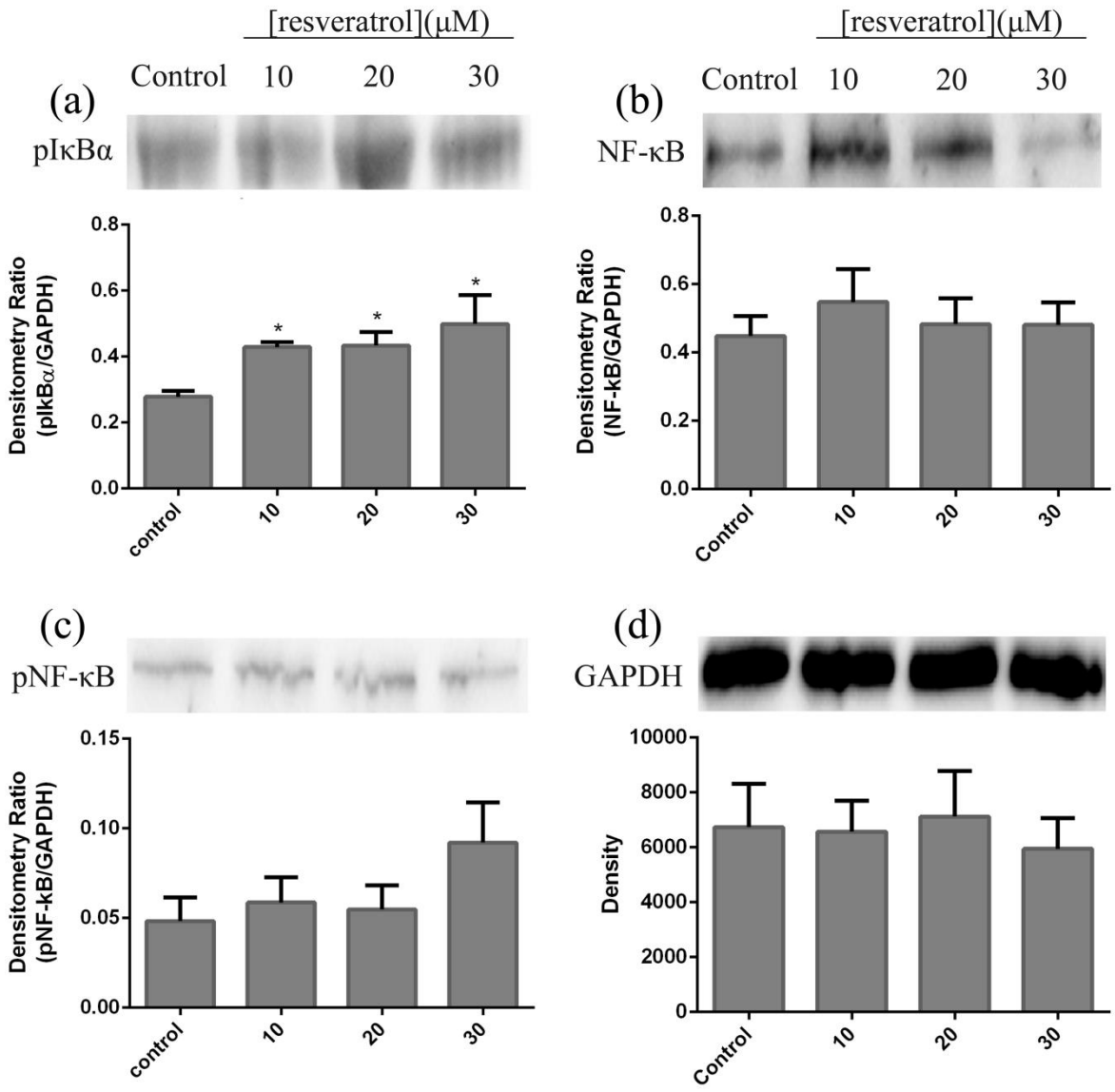

Figure 5.14. Effects of resveratrol treatment for 4 days on SKOV-3 spheroids/clusters. Western blot and densitometry ratios of (a) pIκBα (b) NF-κB (c) pNF-κB (d) GAPDH. Western blot images are representative of two experiments carried out in duplicate. The glycolytic enzyme GAPDH was used as a reference protein and 10 μg of protein per well was loaded for each sample. Densitometry ratios are relative to GAPDH, whose expression did not change after treatment (d). Results are representative of two independent experiments analysed in duplicate.

5.4 Discussion.

Overall the results show that inhibiting NF-κB does reduce the growth of ovarian cancer spheroids/clusters. Therefore, it was a viable pathway to investigate in regards to the mechanisms underpinning the anti-cancer effects of resveratrol and acetyl resveratrol. This study showed that it is possible that the chemopreventive activity of resveratrol and acetyl resveratrol may in part be due to their ability to reduce total expression of NF-κB. However, this effect is cell line dependent.

5.4.1 Treatment effects on signalling proteins.

Phosphorylation of NF-κB has been shown to increase the transcriptional action of NF-κB (Egan et al. 1999); therefore, in this study I looked for expression of both NF-κB and pNF-κB in the two cell lines to get a clearer indication of the activity of NF-κB. Western blotting revealed that in the SKOV-3 cell line there was a significant dose dependent reduction in NF-κB and pNF-κB expression when treated with resveratrol for 6 days. Immunofluorescent staining revealed a significant amount of NF-κB and pNF-κB located in the cytoplasm and the nucleus of control SKOV-3 cells. Resveratrol treatment appears to reduce the quantity of the proteins overall and there is noticeably less in the nuclei. This could suggest that the NF-κB signalling pathway may be in part responsible for the production of VEGF in this cell line as levels of NF-κB correlate with VEGF secretion. A decrease in NF-κB resulting in diminished cell viability has been identified in other cancers and even monolayer cultures of ovarian cancer (Alberti et al., 2012). However, this may be the first time the correlation has been observed utilising 3D ovarian cancer cultures, providing a possible link between NF-κB signalling and early metastasis of ovarian cancer.

The expression of the NF-κB inhibitor pIκBα was also measured to give an indication as to whether resveratrol decreases NF-κB itself or a protein further upstream in the signalling cascade. Western blot analysis of pIκBα expression in SKOV-3 suggested that NF-κB is the possible target of resveratrol and not pIκBα or other signalling molecules further upstream, as pIκBα expression was not attenuated by resveratrol treatment. Unexpectedly, the overall expression level of pIκBα in SKOV-3 was very low, as phosphorylation of IκBα is purported to be essential for the activation of NF-κB, therefore expression of pIκBα was

expected to be similar to NF-κB. There are a few possibilities for what could be taking place in the cells; firstly, there is evidence that the ubiquitination and degradation of pIκBα occurs rapidly (Karin and Ben-Neriah, 2000), thus after 6 days treatment most may have already been degraded. The expression of pIκBα after 4 days treatment with resveratrol compared to the pIκBα expression after 6 days with both resveratrol and acetyl resveratrol would suggest that rapid degradation of pIκBα is likely as the 4 day expression is at least two fold greater. It has also been suggested that only 10-20% of RelA located in the cytoplasm is coupled to IκBα, indicating that there may be a significant pool of RelA that is potentially regulated by pathways independent of IκBα (Brasier, 2006). Therefore, it is possible that the canonical IκBα pathway is not involved in NF-κB expression in the SKOV-3 cell line. One potential alternative pathway is the conjugation of RelA to either p105 or p100, these are precursors for p50 and p52 respectively, when stimulated the processing of the precursor results in the release of the NF-κB subunits that are then free to translocate to the nucleus (Karin and Ben-Neriah, 2002). This would mean that there was still a high probability that resveratrol affects upstream signalling proteins of an alternate pathway and not NF-κB directly.

Although treatment with acetyl resveratrol did not produce statistically significant suppression of either NF-κB or pNF-κB, the overall trend of the western blot and immunofluorescence results correlate with the resveratrol results. This suggests that acetyl resveratrol may be a viable treatment option.

Whilst the presence of NF-κB was detected in both cell lines, it is expressed to a much greater extent in OVCAR-5 cells than in SKOV-3 cells. However, this high expression of NF-κB did not translate into high secretion of VEGF or IL-8. Although there does appear to be a dose dependent reduction of NF-κB in OVCAR-5 after 6 days treatment with resveratrol, the expression of the protein is still high even after treatment with the highest dose of resveratrol. Immunofluorescent staining of NF-κB and pNF-κB showed no obvious change in the density or distribution of the proteins for the various treatment concentrations. Furthermore, there were limited levels of NF-κB or pNF-κB in the nuclei of the OVCAR-5 cells. This indicates that most of the NF-κB is in the cytoplasm and therefore inactive. Supporting this is the low expression of pIκBα in OVCAR-5 cells. Assuming that the canonical NF-κB pathway is dominant in OVCAR-5, low pIκBα suggests that IκBα may be

intact and supressing NF-κB activation. However, this is by no means definitive as western blots of OVCAR-5 proteins were only performed once due to the lack of response in previous experiments and time limitations, therefore statistical analysis was not possible and the various possibilities regarding IκBα expression in SKOV-3 also apply to OVCAR-5. Surprisingly the proportion of phosphorylated NF-κB compared to non-phosphorylated NF-κB was much higher in OVCAR-5 than in SKOV-3. This was unexpected as phosphorylation of NF-κB is purported to induce transcriptional activity and as noted above high NF-κB expression in OVCAR-5 did not translate into the expression of the two more common products of NF-κB activity, IL-8 and VEGF. However, even though NF-κB's transcriptional activity is increased by phosphorylation it has no effect on translocation of NF-κB (Egan et al., 1999), so increased activity of NF-κB may not automatically be correlated with phosphorylation. There is also the possibility that the detected phosphorylation is actually attached to an unprocessed precursor of p50 or p52 complexed with RelA as phosphorylation of the precursor is the signal for post-translational modification of the precursor, thus a high proportion of inactivated pNF-κB located in the cytoplasm is possible (Gilmore, 2006). Another reasonable explanation for the increased pNF-κB expression is that OVCAR-5 is deficient in IKKβ, which is an upstream kinase in the NF-κB signalling pathway responsible for the nuclear translocation of NF-κB (Sizemore et al., 2002). NF-κB would be phosphorylated by IKKα but then unable to enter the nucleus.

Chapter 6

Conclusions.

6.1 Main conclusions.

The high mortality rate of ovarian cancer is due to a lack of early detection and its uncommon mode of dissemination. Current treatment options are very harsh and there is a high incidence of developed resistance (Lengyel, 2010). Therefore, the need for alternative chemopreventive and chemotherapeutic agents is urgent. Anti-tumour properties have been identified in many natural compounds present in food, of these resveratrol and EGCG have been shown to be quite effective in reducing cancer growth (Athar et al., 2009).

This study has demonstrated that resveratrol and the resveratrol analogue acetyl resveratrol are effective food compounds in reducing the cellular metabolism and growth of certain sub-types of ovarian cancer cells through prolonged exposure to concentrations that are possible for a person to ingest through their daily diet. EGCG on the other hand, does not appear to be able to affect the growth of ovarian cancer. Therefore, resveratrol and acetyl resveratrol are viable candidates for possible treatment of certain sub-types of ovarian cancer.

This investigation has also provided some insight into the mechanisms underlying the anti-cancer property of resveratrol and acetyl resveratrol. This study has shown that NF-κB levels and VEGF secretion correlate to the reduction of cell growth of ovarian cancer treated with resveratrol and acetyl resveratrol. These data suggest that NF-κB signalling may play a role in the secretion of VEGF which in turn possibly sustains cell viability and growth of free floating ovarian cancer cells in the peritoneal cavity. The association of NF-κB and VEGF has also been noticed in clinical patient tumours.

6.2 Future Research.

As EGCG had been shown to be effective in reducing the growth of ovarian and other cancers in monolayer cultures as well as animal models, further work could be undertaken to investigate why it did not affect ovarian cancer spheroids/clusters. One plausible possibility is that the concentration of EGCG used in this study was not high enough to elicit an effect, meaning that the average dietary intake of EGCG would not be effective in reducing the spread of this type of cancer. Another is that EGCG treatment at these dosage levels require a longer time period to be effectual, this would mean that dietary intake of the levels of EGCG utilised in this study may still be beneficial in restricting the metastasis of ovarian cancer.

Due to the resveratrol and acetyl resveratrol effects being cell line specific it would be prudent to test the treatments on a much wider range of cell lines to determine how effectual the treatments are overall in ovarian cancer. Also an investigation into the bioavailability of acetyl resveratrol is now justified. As it is suggested that acetyl resveratrol should be more bioavailable than resveratrol and the analogue has now been shown to be chemotherapeutic in its current structural form.

It is very interesting to note that resveratrol and acetyl resveratrol can reduce VEGF but the cancer cells secrete more IL-8 in response. Both of these peptides are crucial for angiogenic activity in solid tumours. This suggests that cancer cells are possibly compensating for the blocked pathway by producing alternative angiogenic molecules.

Finally, there is more work to be done to better elucidate the mechanisms of resveratrol's effects. The activity of proteins further upstream of pIκBα as well as the abundance of IκBα could be studied in SKOV-3 cells to better understand the true target of resveratrol. Furthermore, proteins involved in other signalling pathways such as the non-canonical NF-κB or could be investigated in OVCAR-5 cells. This would provide insight into the mechanisms of resveratrol resistant ovarian cancer sub-types and aid the development of more effective treatments.

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Appendix.

Table A1: Reagents utilised in this study.

Alamar Blue	Invitrogen, CA, USA
Trypsin-EDTA	Gibco, Life Technologies
MEM	Gibco, Life Technologies
FBS	Gibco, Life Technologies
PenStrep	Gibco, Life Technologies
Glutamax	Gibco, Life Technologies
Fungizone	Gibco, Life Technologies
DMEM	Gibco, Life Technologies
Trypsin powder	Gibco, Life Technologies
EDTA	Gibco, Life Technologies
Crystal Violet	Gibco, Life Technologies
Resveratrol	Biotevia, USA
Acetyl-resveratrol	Biotevia, USA
EGCG	Sigman, New Zealand
Bay 117085 (NF-kB inhibitor)	Santa Cruz, USA
DMSO	BDH Ltd, Poole, England
$NaHCO_3$	BDH Ltd, Poole, England
Tween-20	BioRad Laboratories, CA, USA
BSA	Gibco, Life Technologies
Ultra-TMB	Thermo Scientific, Illinois, USA
NaN_3	BDH Ltd, Poole, England
SDS	BioRad Laboratories, CA, USA
Tris	Invitrogen, CA, USA
Protease inhibitor tablet	Thermo Scientific, Illinois, USA
EDTA	BDH Ltd, Poole, England
Methanol	Ajax Finechem Pty Ltd, NZ
Ethanol	Technical Grade
Concentrated HCl	Ajax Finechem Pty Ltd, NZ
Skim Milk powder	Pam's, Australia
Na_2HPO_4	BDH Ltd, Poole, England
NaH_2PO_4	BDH Ltd, Poole, England
KCl	BDH Ltd, Poole, England
NaCl	Ajax Finechem Pty Ltd, NZ
30% Acrylamide	BioRad Laboratories, CA, USA
TEMED	BioRad Laboratories, CA, USA
$(NH_4)S_2O_8$	BioRad Laboratories, CA, USA

NaOH	BDH Ltd, Poole, England
Glycine	Sigma-Aldrich LTD, NZ
Aniline blue	Sigma-Aldrich LTD, NZ
CryO-Z-T	Ted Pella Inc, USA
p-phenylenediamine	Sigma-Aldrich LTD, NZ
Hoechst 33342	Invitrogen, NZ

Table A2: Primary antibodies used for western blots and immunofluorescence.

Primary	**Type**	**Concentration**	**Blocking Solution**	**Company**
CK-18	Mouse	1/500	5% Skim milk	Santa Cruz Biotechnology, USA
SOD-1	Rabbit	1/500	4% BSA	Santa Cruz Biotechnology, USA
Ribosomal Protein L13A	Mouse	1/500	1% BSA	Santa Cruz Biotechnology, USA
GAPDH	Rabbit	1/1000	5% Skim milk	Santa Cruz Biotechnology, USA
PCNA	Mouse	1/500	5% Skim milk	Santa Cruz Biotechnology, USA
pIκBα	Mouse	1/500	2% BSA	Santa Cruz Biotechnology, USA
NF-κB	Rabbit	1/500	2% BSA	Santa Cruz Biotechnology, USA
pNF-κB	Rabbit	1/500	5% Skim milk	Santa Cruz Biotechnology, USA

Table A3: Secondary antibodies used for western blots.

Secondary	**Type**	**Concentration**	**Company**
Mouse	Donkey	1/10000	Santa Cruz Biotechnology, USA
Rabbit	Goat	1/10000	Cell Signalling Technology, USA

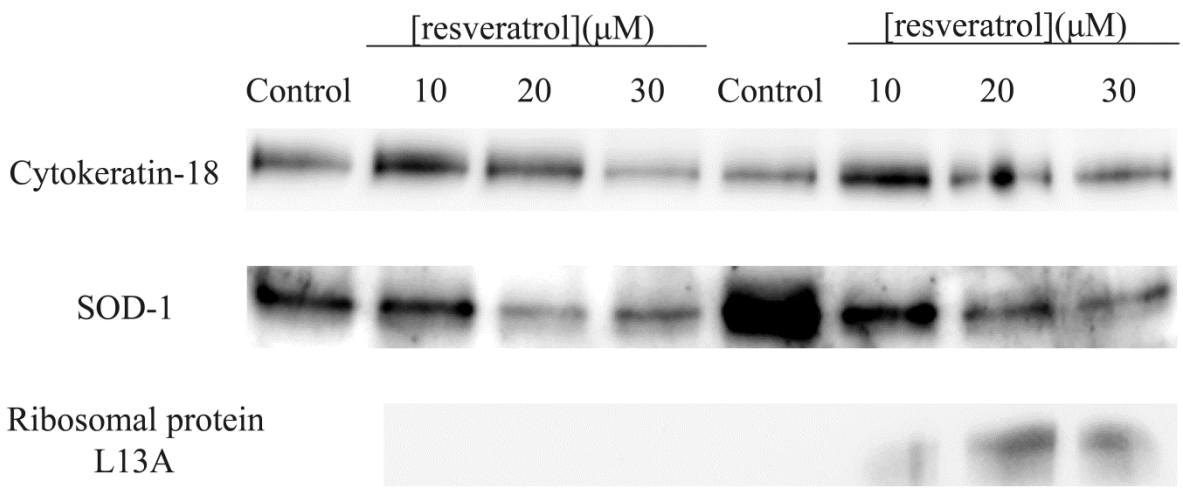

Figure A1: Trial of other potential housekeeping proteins. Images are representative of two trials carried out in duplicate. 10μg of protein per well was loaded for each sample. Samples from the 6 day resveratrol treatments were used for the trial.